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INTRODUCTION

Breast cancer is the second leading cause of cancer death in women and incidence rates continue to rise. While survival rates of women diagnosed with early stages of breast cancer have significantly improved, the survival rates of women with more advanced disease remain quite poor. Thus, novel diagnostic, prognostic, and therapeutic modalities are needed. Metastasis is the primary cause of fatality in breast cancer patients and is believed to begin with the loss of cell adhesion in neoplastic epithelium. Although tight junctions (TJs) have clearly been shown to play a role in cell adhesion, their potential role in cancer progression has been scarcely studied. In 1998, a new family of TJ proteins named Claudins (CLDNs) was discovered (1, 2), which are now known to be the main sealing proteins of the TJ. Although changes in tight junction permeability have been observed in several types of cancer, little is known about the role of CLDNs in cancer. Despite the high degree of sequence similarity among the 20 CLDN family members there is evidence suggesting that each CLDN may have a unique biological function. For example, CLDN 1-deficient mice have been shown to die within 1 day of birth despite the presence of CLDN 4, while CLDN 3 and 4 alone serve as receptors for *Clostridium perfringens* enterotoxin (CPE). This proposal arose out of observations that several members of the claudin gene family were differentially expressed in invasive ductal carcinomas of the breast (3). Compared to normal breast, Claudin-7 expression was lost in 50% of breast carcinomas, while Claudin 3 and 4 were generally overexpressed in breast carcinomas (3).

BODY:

Statement of Work and accomplishments, 2001-2004

Task 1. (months 1-6). Generate clones of Claudin-7, tagged and untagged with marker genes, transfect cells, obtain stable clones in all three cell lines, MCF-7, SKBR3, and MDA-MB-435. Confirm the increased expression of Claudin-7 in transgenic cells and solid tumors derived from transgenic cells.

This task was carried out throughout the period of the grant, due to the technical difficulties encountered with expressing Claudin 7 in breast cancer cells.

Year 1:

We were unable to generate stable clones expressing CLDN-7 protein in the CLDN-7-deficient human breast cancer cell lines MDA-MB-231 and MDA-MB-435. Therefore, we attempted to generate stable clones in the CLDN-7 deficient cell line MCF-7ADR. Similar to our experience with MDA-MB-231 and MDA-MB-435, stable transfection of CLDN-7 cDNA into MCF-7ADR cells yielded CLDN-7 mRNA, but no CLDN-7 protein was generated. This difficulty appears to be unique to CLDN-7 as both CLDN-3 and CLDN-4 protein can be detected following transfection of all CLDN-7 deficient breast cancer cell lines tested using the same vector. Further, CLDN-7 protein can be obtained following transfection of CLDN-7 deficient human mammary epithelial cell lines HBL-100 and MCF-10A, suggesting that the CLDN-7 construct is functioning properly. We next tested the possibility that CLDN-7 protein was folded in the CLDN-7 deficient breast cancer cell lines such that the epitope recognized by our CLDN-7 antibody was not accessible. To test this we generated a construct containing CLDN-7 fused to EYFP at the C-terminus. Consistent with our previous findings we were not able to detect CLDN-7 protein using our CLDN-7 antibody following transient transfection of MDA-MB-435 cells with the CLDN-7-EYFP fusion construct. However, the CLDN-7-EYFP fusion protein was detected both by fluorescence and Western blotting with EYFP antibody. Unfortunately, the protein was not localized to the cell membrane, suggesting that the presence of the EYFP interferes with the proper localization of CLDN-7. To address this issue we then generated a construct containing

CLDN-7 fused to a FLAG tag at the N-terminus. The small size of the FLAG tag would allow us to detect CLDN-7 protein using the FLAG antibody having little chance of interfering with CLDN-7 localization. Interesting, we were not able to detect the FLAG-CLDN-7 fusion protein following transient transfection of MDA-MB-435 cells, although we were able to detect the expression of other FLAG tagged proteins. The fact that the presence of EYFP at the C-terminus of CLDN-7 allowed expression of the protein while a FLAG tag at the N-terminus did not suggests that the C-terminus may be important for the regulation of CLDN-7 expression. Although CLDN proteins have a high degree of sequence similarity, the C-termini of CLDNs are relatively unique. Interestingly, the C-terminus of CLDN-7 contains several unique phosphorylation sites compared to CLDN 3 and 4, which may be involved in the regulation of CLDN-7 protein expression in MDA-MB-231, MDA-MB-435, and MCF-7ADR cells. We will conduct further studies to determine the cause of this differential regulation, which may provide some insight into the regulation of CLDN-7 in breast cancer.

Year 2: To circumvent this problem we generated stable clones expressing CLDN-7 in the CLDN-7-deficient human mammary epithelial cell line, HBL-100. Although HBL-100 is considered to be phenotypically normal at early passage numbers, it has been reported to acquire invasive properties at later passages. We confirmed the expression of CLDN-7 protein in our stable clones expression CLDN-7, HBL-100(7), by Western blotting and immunofluorescence microscopy. None of the HBL-100 cells, HBL-100 (WT-wild type), HBL-100(VC-vector control), and HBL-100(7) were able to grow in soft agar.

We next looked for evidence of reduced malignant phenotype of HBL-100(7) cells versus HBL-100(WT) and HBL-100(VC) cells by culture in Matrigel. The metastatic breast cancer cell line MDA-MB-231 and the immortalized normal mammary epithelial cell line MCF-12A were included as examples of invasive and normal growth, respectively. All cells were grown on the surface of Matrigel and media was replenished every other day. After one day organized ductal structures and acini were formed by all HBL-100 cell lines and MCF-12A, while MDA-MB-231 cells formed acini alone. After two days the ductal structures and acini in HBL-100(WT), HBL-100(VC), and MDA-MB-231 cultures began to form extensions, while ductal structures in HBL-100(7) and MCF-12A cultures regressed and the acini remained dormant. The extensions observed from ductal structures and acini in HBL-100(WT), HBL-100(VC), and MDA-MB-231 cultures continued to grow out over the 7 day experiment while ductal structures disappeared and acini remained dormant in HBL-100(7) and MCF-12A cultures. This suggests that CLDN-7 expression did not affect the initial formation of ductal structures, but did prevent further cell growth. While MDA-MB-231 cells formed similar structures in Matrigel to those seen in HBL-100(WT) and HBL-100(VC) cultures, HBL-100(7) cells had a similar appearance to MCF-12A cells. These data provide evidence to suggest that CLDN-7 expression can suppress the invasive phenotype of HBL-100 cells grown in Matrigel.

HGF/SF is known to induce cell spreading and induce the outgrowth of mammary ductal epithelium. Due to the ability of HGF/SF to stimulate cell growth and reduce cell adhesion, it is believed to play a role in breast cancer development. In a separate experiment the same cell cultures were treated with HGF/SF one day following seeding on Matrigel. HBL-100(WT) and HBL-100(VC) cells treated with HGF/SF formed extensions much faster than in untreated cells, consistent with the biological function of HGF/SF. HBL-100(7) cells were resistant to the growth inducing effects of HGF/SF, although some extension formation could be seen. These data further support a growth suppressing effect of CLDN-7 expression in HBL-100 cells when cultured on Matrigel. We attempted to determine the effect of CLDN-7 on invasion in vivo using HBL-100 cells, but they did not grow in nude mice.

Year 3: We have devoted effort to understanding why CLDN-7 expression is excluded from some breast cancer cell lines and not in others. The pattern of expression appears to be that the cell lines that grow as noninvasive tumors in immunodeficient mice, such as MCF-7 cells, allow the expression of exogenously expressed CLDN-7, while cell lines that form invasive tumors and metastasize to the lungs in immunodeficient mice, such as MDA MB 231 and MDA MB435, do not. In each of the latter cell lines exogenously introduced CLDN-7 mRNA is expressed, while the protein is not. We have investigated the molecular basis of this difference further.

First, we confirmed that the 231 and 435 cell lines do not have a generalized inability to express Claudins. We found that both were able to express CLDN-3 and 4 with an efficiency equivalent to MCF-7 cells. Next, to determine if the amino acid sequence of these proteins would provide insight into what might contribute to the inability to be expressed in 231 and 435 cells, we compared the sequences of CLDN-3, and 4 with that of CLDN-7. All claudin family members share a great deal of similarity at the amino acid level. However, CLDN-3 and 4 were found to be very similar to each other, while CLDN-7 is a relatively distant relative, and more similar to CLDN-1. More precisely, the dissimilarity is marked at its N-terminal end.

We reasoned that the N-terminal end may contribute to the greater stability of CLDN-3 and 4 compared to CLDN-7. A number of constructs were made swapping the N-terminal (signal peptide), and larger portions of CLDN-7 into CLDN-3, and vice versa. Replacing CLDN-3 signal peptide sequences into the CLDN-7 expression constructs, resulted, upon transfection, in the generation of stable chimeric protein. The converse was also true- placing CLDN-7 signal peptide into CLDN-3 resulted in the inability to detect protein in those cells. Greater the contribution of CLDN-3 sequences to the construct, higher the level of the detected protein. Further definition of these sequences is underway. However, it is clear that the level of protein in 231 and 435 cells never equals the level expressed in the permissive MCF-7 cells. Thus, in addition to the amino acid sequence, it is likely that the 231 and 435 cells degrade the protein, or contain some deleterious factors that do not permit detection of CLDN-7 in these cells. To address this question, we must first determine if CLDN-7 protein is synthesized. If so, is it degraded, and by what pathway? Pulse chase experiments are underway as a first step to address these questions.

CLDN-7 is a tight junction protein, and our work has shown that CLDN-7 expression is lost in poorly differentiated carcinomas, and in lobular carcinoma in situ. Loss of this Claudin may be critical to invasion and metastasis. We have been unable to express CLDN-7 in a number of metastatic human and mouse breast cancer cell lines. It is interesting to speculate that the metastatic cells devise a mechanism to rapidly degrade CLDN-7 to acquire a more mesenchymal phenotype, and maintain their ability to invade other organs. Once the tumor cells settle in that organ, there is a reversion from mesenchymal to epithelial phenotype and CLDN-7 is re-expressed. Our further work will shed light on this important question.

CLDN 3 and 4

Year 2:

Due to the previously described technical difficulties encountered in the proposed Statement of Work, we began studying the expression and biological role of CLDN 3 and 4 in breast cancer. CLDN 3 and 4 are reportedly over-expressed in several carcinomas. CLDN 3 and 4 are unique among CLDNs in that they function as receptors for the *Clostridium perfringens* enterotoxin (CPE), the causative agent of diarrheal symptoms following *Clostridium perfringens* type A food poisoning. The symptoms are initiated when CPE binds to CLDN 3 and 4, leading to the formation of membrane pore complexes and subsequent cytolysis of intestinal epithelial cells. We determined the expression of CLDN 3 and 4 in breast cancers and the potential of CPE-mediated therapy. By Western analysis, CLDN 3 and 4 were

expressed in 9/9 primary breast carcinomas and 7/10 breast cancer cell lines tested at levels similar to that in normal mammary epithelial cells, contrary to the results of the SAGE analysis. Immunohistochemical analysis of primary breast carcinomas showed that CLDN 3 and 4 were detectable solely in epithelial cells and were localized to the cell membrane. Treatment of breast cancer cell lines expressing CLDN 3 and 4 with CPE for 60 minutes in vitro resulted in dose-dependent cytolysis, while cells not expressing CLDN 3 and 4 remained unaffected. Further, intratumoral administration of CPE (10 ug) to xenograft tumors of T47D breast cancer cells in immunodeficient mice resulted in a significant reduction ($p = 0.007$) in tumor volume concurrent with extensive necrosis as determined by Hematoxylin and Eosin staining. This phenomenon was also observed upon treatment of three freshly resected primary breast tumor tissues with CPE (10 ug) in vitro. Further, CPE (2 ug/ml) treatment of primary breast carcinoma cells, purified by immunobead selection, resulted in nearly 100% cell death within 60 min. Thus, the expression of CPE receptors CLDN 3 and 4 in primary breast carcinomas sensitizes them to CPE-mediated cytolysis and emphasizes the potential of CPE in breast cancer therapy. Strikingly, CPE injected into the duct of normal rats affects no change in their morphology. This result suggests that CPE is effective only when claudins 3 and 4 are expressed at robust levels in the cells. The low levels of the two proteins seen in normal luminal epithelium does not appear to be sufficient for the penetration and internalization of toxic amounts of the drug. Another possibility is that the tight junctions between luminal epithelial cells is very tight, and does not permit binding of sufficiently high levels of CPE to these cells to cause lytic death. The paper describing these findings was published this year in American Journal of Pathology, and is appended. Our recent work on MNU-induced mammary tumors indicates that intraductal injection of CPE to ducts bearing palpable tumors results in regression of the tumors and long term survival. Slow release formulations will allow better utility of this agent and this mode of delivery.

Year 3:

Our work has been extended into determining if metastasis of breast carcinomas at the brain and bone express CLDN3 and 4, and whether CPE therapy would be an effective treatment for distant metastasis. Examination of a panel of 15 breast cancer metastasis to the brain and bone by IHC shows that the majority of the metastatic tumors expressed both CLDN3 and 4. Next, we confirmed that these claudins are not expressed in the host tissue. All parts of the brain showed no expression of CLDN3 and 4 by IHC. The same was true for the multiple cell types present in the bone. This raised the possibility of treating these distant metastasis using CPE. To this end, we developed a nude mouse model of brain metastasis by injecting MDA MB 468 cells into the brain of mice by stereotactic injection. Treatment of these mice by injection of CPE into the brain prolongs the life of the mice by two fold. Slow release formulations using polymer wafers, polymer beads and nanoparticles is ongoing to provide a longer lasting effect of CPE. No adverse effects on brain function or histology as a result of treatment was noted. No systemic toxic effects were seen. This studies are being actively pursued in more detail.

Task 2 : (months 3 -12): Study t he T ER, e nzymatic d ispersion a nd r eaggregation c haracteristics of Claudin-7 transfectants compared to parental cells.

In Task 2, we attempted to determine the impact of CLDN-7 expression on tight junction structure and function. Task 2 could not be performed as HBL-100 cells, the only cells that stably express CLDN7 do not form a uniform monolayer that is required to perform transepithelial electrical resistance (TER) and paracellular permeability assays.

Task 3. (months 5-17). Characterize invasive behavior and metabolic profiles of control and transgenic cells for MCF-7, SKBR3 and MDA-MB-435 cells.

Since generating stable lines of CLDN7 expressing breast cancer cells has presented a difficult problem, we are proposing to use siRNA technology to suppress expression of endogenous CLDN7 in SKBR3 cells and determine if the cells become more tumorigenic and invasive. SKBR3 breast cancer cells form avascular tumors with large number of cells (10 million) and with long latency (5-7 weeks) when injected into nude mice. These cells express easily detectable CLDN7. Stably transfectable siRNA plasmids will be engineered to obtain clones with loss of CLDN 7 expression. These cells will be injected into SCID mice. If tumor growth rate and appearance is changed, animals will be examined for development of metastasis in the lungs. The tumors will then be examined by MRI in Task 4.

KEY RESEARCH ACCOMPLISHMENTS:

1. Characterization of CLDN-7, -3 and -4 expression in breast carcinomas. CLD-7 expression was lost in the majority of invasive ductal carcinomas, and more markedly in poorly differentiated tumors. Loss of CLDN-7 marks the acquisition of a more mesenchymal (invasive) phenotype. CLDN-3 and 4 are overexpressed in nearly 75% of breast carcinomas.
2. Cells that are invasive and metastatic do not express CLDN-7, while non invasive breast cancer cell lines express CLDN-7. More importantly, the invasive cells do not allow expression of exogenously introduced CLDN-7 expression constructs. Nonpermissive cells could contain factors that degrade CLDN-7, and need to be investigated.
3. CLDN3 and 4 are receptors for the bacterial toxin Clostridium perfringens exotoxin (CPE). Intratumoral injection of CPE results in tumor regression. Intraductal injection of CPE results in regression of MNU induced tumors, with no side effects on normal epithelial cells.
4. CLDN3 and 4 are abundantly expressed in both brain and bone metastasis of breast carcinomas. Survival of mice bearing xenografts of human breast carcinoma cells is doubled by intratumoral treatment with CPE.
5. Labeled antibodies to CLDN3 and 4 can be used for imaging tumors. The antibody concentrates in the xenografted breast tumor and is visualized easily by MR.

REPORTABLE OUTCOMES:

Manuscripts:

Kominsky SL, Argani, P, Korz D, Evron E, Raman V, Garrett E, Rein A, Sauter G, Kallionemi O-P, Sukumar S. Loss of tight junction protein Claudin 7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. Oncogene, 22: 2021-2033, 2003.

Kominsky SL, Mustafa V, Korz D, Gabig TG, Argani P, Sukumar S. *Clostridium perfringens* enterotoxin elicits rapid and specific cytolysis of breast carcinoma cells mediated through tight junction proteins claudin 3 and 4. Amer J Pathol, 164:1627-1633, 2004.

Presentations:

June 2004: "SAGE analysis of Breast Cancer- from bench to bedside"- from ICMIC Center in Johns Hopkins

June, 2004: National Cancer Institute "Exploiting Gene Alterations in Breast Cancer"

April, 2004: University of Alabama at Birmingham "Intraductal approaches to breast cancer detection, prevention and therapy"

April 2004: ""Intraductal approaches to breast cancer detection, prevention and therapy"
The Normal Breast Think Tank, Santa Barbara, CA

April 2004: AACR meetings, Orlando, Florida.

June 12, 2003, Howard/Hopkins Partnership Steering Committee Meeting, Howard University Cancer Center, Washington, D.C. "Comparative Gene Expression Analysis in African American and Caucasian Breast Cancer"

March 27-30, 2003, 3rd International Santa Barbara Symposium, The Intraductal Approach to Breast Cancer, Santa Barbara, California, "Detection of Breast Cancer Cells in Ductal Lavage and Blood Using Hypermethylated Gene Markers"

January 7-8, 2003, Institute of Medicine Committee on New Approaches to Early Detection and Diagnosis of Breast Cancer. Workshop on New Approaches to Breast Cancer Detection, Washington, D.C., "The Search for Breast Cancer Biomarkers"

January 22-23, 2003, National Council for Johns Hopkins Medicine, Meeting and Reception, Palm Beach, Florida, "Breast Cancer Research – Dawn of a New Era"

September 25–28, 2002 ,Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orange County Convention Center, Orlando, Florida, "Early Detection of Breast Cancer by Molecular Analysis of Ductal Lavage Fluid"

September 19 – 21, 2002, Mildred Scheel Cancer Conference, Frankfurt, Germany, "Gene expression profiling of breast cancer – from bench research to early detection markers"

Patent application – Claudins as markers for early detection, diagnosis, prognosis and targets of therapy for breast and metastatic brain or bone cancer. International Application No. PCT/US03/04371

CONCLUSIONS:

Claudins are transmembrane proteins that seal tight junctions, and are critical for maintaining cell-to-cell adhesion in epithelial cell sheets. However, their role in cancer progression remains largely unexplored. We have reported that Claudin-7 expression is lost in invasive ductal carcinomas (IDC) of the breast, occurring predominantly in high-grade (Nuclear and Elston grade 3) lesions. This pattern of expression is consistent with the biological function of Claudin-7, as greater discohesion is typically observed in high-grade lesions. In summary, these studies provide insight into the potential role of Claudin-7 in the progression and ability of breast cancer cells to disseminate, and suggest that Claudin-7 may be valuable as a prognostic indicator for breast cancer. Future work will address the dissection of the role of Claudin 7 in invasion and metastasis using cell culture, animal model and imaging systems.

We also explored the use of Claudin 3 and 4 overexpression in breast cancer as a target for a bacterial toxin, the Clostridium perfringens enterotoxin. We have shown that breast cancer cells die within minutes of exposure to CPE, and will perform in vivo experiments to take this concept for further development into clinical application. We believe that CPE will provide a very potent antitumor drug. Formulations to increase its effectiveness and slow release are under preparation.

So what?? The proposed studies will identify physiological and metabolic alterations associated with Claudin-7, -3 and -4. A new role for a little known class of proteins, which are components of the tight junctions, will be established. The function of Claudins 3 and 4 as receptors for a potent bacterial toxin allows us to test its potential as an anticancer agent when delivered through ductal opening at the teat. Such physiological and metabolic characterizations will provide rationales for therapeutic designs to prevent metastasis, as well as provide MR detectable risk factors for evaluating disease aggressiveness.

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2. Morita, K., Furuse, M., Fujimoto, K., Tsukita, S. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc. Natl. Acad. Sci (USA) 96: 511-516, 1999.
3. Nacht M., Ferguson, A. T., Zhang, W., Petroziello, J.M., Cook, B.P., Gao, Y.H., S. Maguire, D.Riley, G. Coppola, G.M. Landes, S.L. Madden, and Sukumar, S. Combining SAGE and array technologies to identify genes differentially expressed in breast cancer. Cancer Res. 59: 5464-5470, 1999.

APPENDICES:

2 papers, Curriculum vitae

Oncogene (2003) 22, 2021–2033

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Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma *in situ* and invasive ductal carcinoma of the breast

Scott L Kominsky[1], Pedram Argani[2], Dorian Korz[1], Ella Evron[1,8], Venu Raman[4], Elizabeth Garrett[3], Alan Rein[5], Guido Sauter[6], Olli-P Kallioniemi[7] and Saraswati Sukumar*[,1]

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Claudins are transmembrane proteins that seal tight junctions, and are critical for maintaining cell-to-cell adhesion in epithelial cell sheets. However, their role in cancer progression remains largely unexplored. Here, we report that Claudin-7 (CLDN-7) expression is lower in invasive ductal carcinomas (IDC) of the breast than in normal breast epithelium, as determined by both RT–PCR (9/10) and Western analysis (6/8). Immunohistochemical (IHC) analysis of ductal carcinoma *in situ* (DCIS) and IDC showed that the loss of CLDN-7 expression correlated with histological grade in both DCIS ($P<0.001$, $n=38$) and IDC ($P=0.014$, $n=31$), occurring predominantly in high-grade (Nuclear and Elston grade 3) lesions. Tissue array analysis of 355 IDC cases further confirmed the inverse correlation between CLDN-7 expression and histological grade ($P=0.03$). This pattern of expression is consistent with the biological function of CLDN-7, as greater discohesion is typically observed in high-grade lesions. In line with this observation, by IHC analysis, CLDN-7 expression was lost in the vast majority (13/17) of cases of lobular carcinoma *in situ*, which is defined by cellular discohesion. In fact, inducing disassociation of MCF-7 and T47D cells in culture by treating with HGF/scatter factor resulted in a loss of CLDN-7 expression within 24 h. Silencing of CLDN-7 expression correlated with promoter hypermethylation as determined by methylation-specific PCR (MSP) and nucleotide sequencing in breast cancer cell lines (3/3), but not in IDCs (0/5). In summary, these studies provide insight into the potential role of CLDN-7 in the progression and ability of breast cancer cells to disseminate.

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Introduction

Metastasis is the primary cause of fatality in breast cancer patients. Although there are believed to be numerous events contributing to the process of metastasis, it is widely accepted that the loss of cell-to-cell adhesion in neoplastic epithelium is necessary for invasion of surrounding stromal elements and subsequent metastatic events. Cell-to-cell adhesion in epithelial cell sheets is maintained mainly through two types of junctions: adherens junctions and tight junctions. Numerous studies have focused their attention on the transmembrane protein of the adherens junction, E-cadherin. These studies have shown that impairing the function of E-cadherin can cause cell dispersion and confer invasive properties in various cell types. Owing to these abilities, E-cadherin is believed to function as a tumor suppressor in numerous tissues and has been shown to be a useful prognostic indicator for some tumors, illustrating the importance of cell-to-cell adhesion proteins in cancer progression (Soler *et al.*, 1995; Wheelock *et al.*, 2001).

Tight junctions, unlike adherens junctions, are solely involved in cell-to-cell adhesion and serve two main functions in epithelial cell layers. First, they prevent the paracellular transport of solutes and ions, maintaining concentration gradients driving transcellular transport. Second, tight junctions prevent the diffusion of membrane proteins and lipids from the apical layer to the basolateral layer of an epithelial cell sheet, helping to maintain cell polarity (Mitic and Anderson, 1998). Although tight junctions have clearly been shown to play a role in cell-to-cell adhesion, their potential role in cancer progression has been scarcely studied. This may be due, in part, to the lack of knowledge concerning the protein components of these junctions. However, in

1998, Tsukita *et al.* discovered a new family of tight junction proteins named Claudins (CLDNs) (Furuse *et al.*, 1998). Currently, there are 20 known members of the CLDN family (Mitic *et al.*, 2000).

CLDNs contain four transmembrane domains and two extracellular loops through which they bind to CLDNs on adjacent cells (Morita *et al.*, 1999). CLDNs have also been shown to bind to the tight junction protein ZO-1 through their carboxyl terminus (Itoh *et al.*, 1999). Interestingly, ZO-1 is believed to interact with several proteins involved in cell signaling and transcriptional regulation (Balda and Matter, 2000; Mitic *et al.*, 2000). These studies suggest that CLDNs may play an indirect role in cell signaling and transcriptional regulatory events. Most importantly, studies have shown CLDNs to be the main sealing proteins of the tight junction (Tsukita and Furuse, 1999).

Although changes in the permeability of tight junctions have been observed in several types of cancer, little is known about the role of CLDNs in cancer. In one such investigation, CLDN-1 cDNA levels were found to be decreased in a number of breast tumors and breast cancer cell lines (Kramer *et al.*, 2000). Kramer *et al.* (2000) went on to examine the genetic status of CLDN-1 in a large number of sporadic and hereditary breast cancers, but found no genetic alterations that could explain this loss or provide evidence supporting the involvement of aberrant CLDN-1 in breast tumorigenesis.

Here we present, for the first time, data showing that expression of the tight junction protein CLDN-7 is lost in ductal carcinoma *in situ* (DCIS), lobular carcinoma *in situ* (LCIS), and invasive ductal carcinoma (IDC) of the breast relative to normal mammary epithelium. Loss of CLDN-7 closely associates with the discohesive architecture typically observed in high-grade lesions, suggesting a potential functional role for CLDN-7 in breast cancer progression. While the mechanism of loss of CLDN-7 in breast cancer cell lines could be ascribed to promoter hypermethylation (Jones and Baylin, 2002), this was not found to be the case in primary IDCs. Taken together, these studies suggest that the loss of CLDN-7 may aid the dissemination of cancer cells.

Results

Expression of CLDN-7 mRNA in IDC and normal mammary epithelium

A SAGE and cDNA microarray analysis performed previously in our laboratory had suggested that CLDN-7 was overexpressed in breast cancer cell lines and IDCs of the breast relative to cultured finite lifespan human mammary epithelial cells (HMEC) (Nacht *et al.*, 1999). We initiated validation studies by directly comparing the expression of a number of differentially expressed mRNAs in IDCs using semiquantitative RT–PCR analysis. Confirming data from microarray analysis (Nacht *et al.*, 1999), CLDN-7 expression was undetectable by RT–PCR in finite lifespan HMECs expanded in tissue culture, 16637 and 04372 (Figure 1), and in HMEC 184, 184A1 (early and late passage) and 184B1 (data not shown). Contrary to our expectation, however, easily detectable to high levels of CLDN-7 mRNA expression were seen in seven of seven uncultured luminal and myoepithelial cell populations derived from normal mammoplasty specimens. Also, nine of 10 IDCs showed low or undetectable levels of CLDN-7 mRNA (Figure 1a). These observations were in direct contrast to our published data (Nacht *et al.*, 1999), where we had reported that at least 50% of primary tumors express levels of CLDN-7 mRNA that were significantly higher than cultured finite lifespan HMEC.

One possible explanation for these contradictory findings could be the choice of HMEC used to compare expression profiles between normal and tumor samples. In our study, as in many other comparative gene expression profiling studies (Fujii *et al.*, 2002; Iacobuzio-Donahue *et al.*, 2002), we had used mortal HMEC expanded in tissue culture as our source of normal breast epithelium. We considered the possibility that placing the cells in tissue culture, albeit short term, may have altered their expression profile and resulted in a loss of CLDN-7 expression. To test this possibility, we determined the expression of CLDN-7 in two immortalized and four finite lifespan HMEC cultured *in vitro*, six uncultured HMEC derived from three normal mammoplasty specimens, and 10 breast cancer cell lines by real-time PCR analysis (Figure 1b). A striking difference in CLDN-7 mRNA expression was observed between the six tissue cultured cell lines ($n=2$) and strains ($n=4$) and the six uncultured HMEC. HMEC cultured *in vitro* showed very low to undetectable levels of CLDN-7 mRNA expression, while an average of nearly 1000-fold higher levels were observed in uncultured HMEC, of both luminal and myoepithelial subfractions. Thus, the erroneous conclusion of CLDN-7 overexpression in primary tumors likely arose as a consequence of using cultured HMEC (which expressed extremely low levels of CLDN-7 mRNA) as a basis for comparison. Relative to CLDN-7 mRNA levels in uncultured HMEC, however, CLDN-7 expression in all 10 breast cancer cell lines was lower by 10–1000-fold. Thus, although immaterial for many other genes (Ferguson *et al.*, 2000; Evron *et al.*, 2001a, b; Loeb *et al.*, 2001), placing HMEC in tissue culture had the profound effect of silencing CLDN-7 expression. When used as controls for comparative gene expression studies, such tissue-culture-based alterations could lead to inaccurate interpretation of data.

Generation and characterization of CLDN-7 polyclonal antibody

To study expression of CLDN-7 protein in breast tissues, we generated a rabbit polyclonal antibody against a peptide corresponding to the C-terminus of CLDN-7. This region of the protein shares little sequence similarity with other members of the CLDN family. Next, we used the C-terminal CLDN-7 peptide

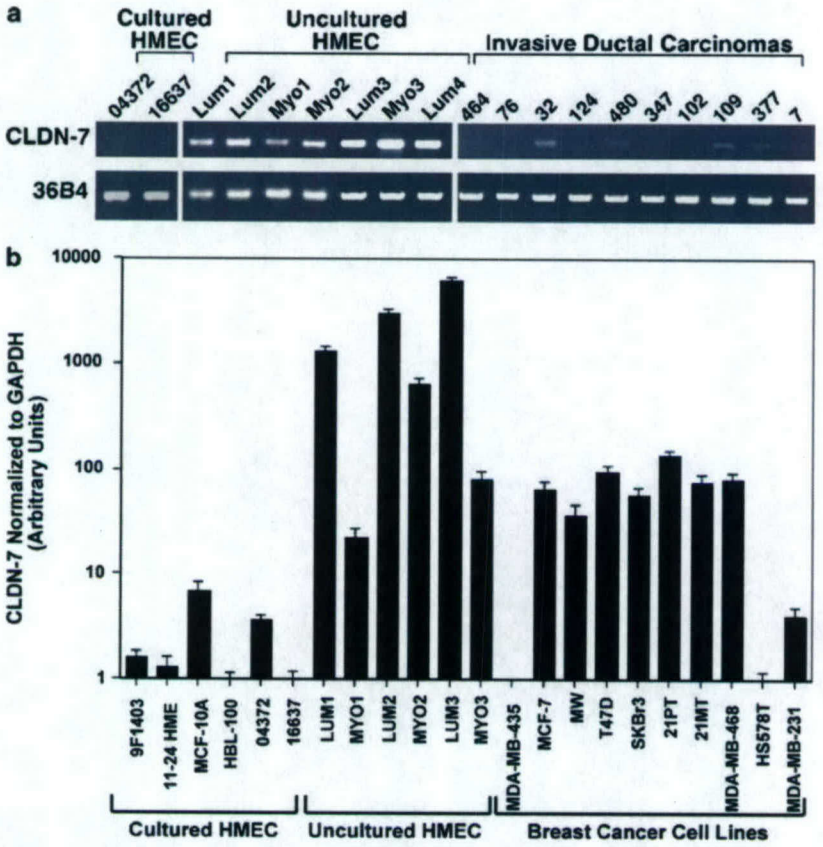

Figure 1 CLDN-7 mRNA expression in breast epithelial cells and primary invasive ductal carcinomas. (**a**) Total RNA was extracted and cDNA was generated by reverse transcription. CLDN-7 and 36B4, a 'housekeeping' ribosomal protein gene, were amplified individually by PCR. PCR products were resolved by electrophoresis on a 1.5% agarose gel. Samples are 16637- and 04372-cultured HMECs from Clonetics; Lum 1-4 and Myo 1-3- Immunobead purified luminal and myoepithelial cells from normal mammoplasty specimens; 10 invasive ductal carcinomas. (**b**) Total RNA was extracted from the indicated cells and cDNA was generated by RT. CLDN-7 and GAPDH (a 'housekeeping' gene) were amplified individually by real-time PCR. CLDN-7 expression levels were normalized to levels of GAPDH, multiplied by 10^2, and reported in arbitrary units $\pm$ s.d. Data are from experiments performed in triplicate

in enzyme-linked immunosorbent assay (ELISA) to test for the presence of CLDN-7 antibody in rabbit sera, and affinity purified the CLDN-7 antibody using the peptide against which it was raised. Western analysis of CLDN proteins generated *in vitro* showed that the antibody reacted with CLDN-7 while showing no crossreactivity with CLDN-3 or -4 (Figure 2a). Conversely, antibodies to CLDN-3 and -4 did not detect CLDN-7 protein, but detected their cognate protein. Further, preincubation of CLDN-7 antibody with the C-terminal peptide was able to compete out binding to CLDN-7 protein in Western analysis (data not shown). Immunofluorescence studies using the affinity purified antibody showed colocalization of CLDN-7 with the tight junction protein ZO-1 at the cell membrane (Figure 2b). Whether the unique red spots in the cytoplasm represent nonspecific staining or CLDN-7 localized in cell organelles is not yet known. These spots were not localized to mitochondria, however, since they did not colocalize with organelles stained by using the MitoTracker Red dye (Molecular Probes, Eugene ORE).

Expression of CLDN-7 protein in IDC and normal mammary epithelium

To determine whether protein expression reflected that of CLDN-7 mRNA expression as obtained by RT–PCR, we performed Western analysis on a panel of 10 breast cancer cell lines, eight IDCs, and four samples of mammary organoids isolated from reduction mammoplasty specimens of normal women. Consistent with real-time quantitative RT–PCR results (Figure 1b), Western analysis of a panel of 10 breast cancer cell lines showed a close correlation between CLDN-7 protein (Figure 3a) and CLDN-7 mRNA expression. Cell lines that showed low or no detectable mRNA (MDA-MB-435, MDA-MB-231, and HS578T) had no detectable protein, while the remaining seven cell lines showed detectable CLDN-7 expression (Figure 3a). Also consistent with RT–PCR (Figure 1a), CLDN-7 expression in six of eight IDCs was significantly lower than in four samples of epithelial organoids obtained by enzymatic digestion of normal mammoplasty specimens (Figure 3b).

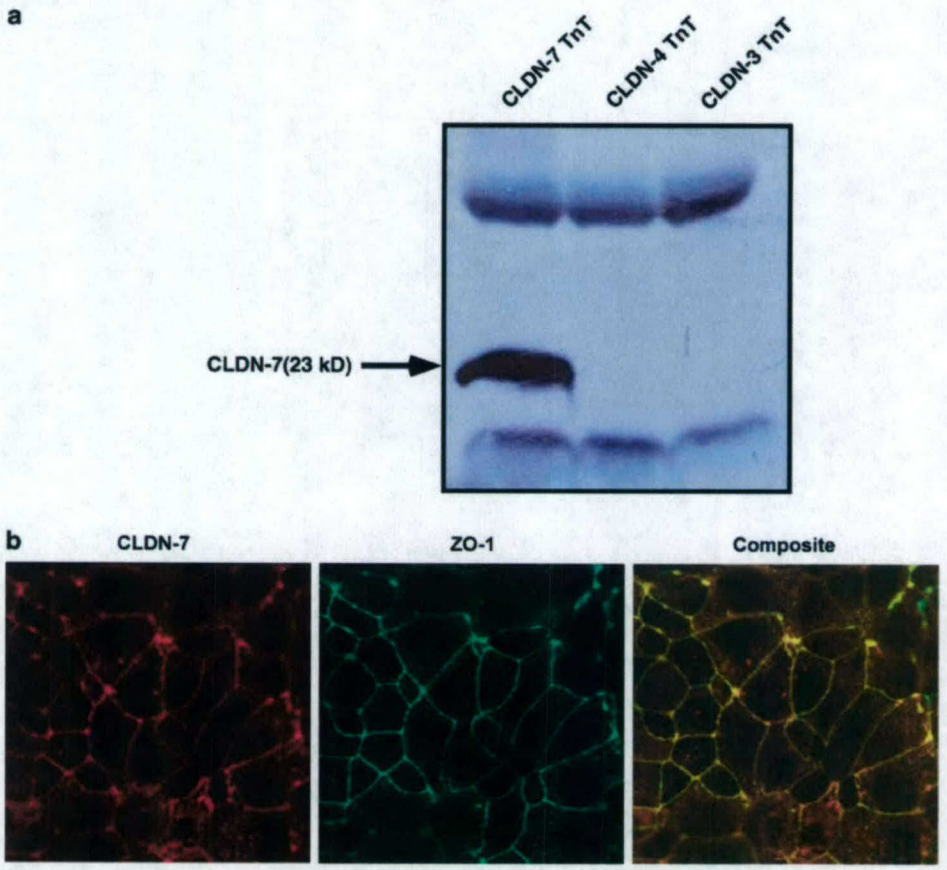

Figure 2 Characterization of CLDN-7 polyclonal antibody. (**a**) Affinity-purified CLDN-7 pAb reacts with human CLDN-7 protein, while showing no crossreactivity with human CLDN-3 or -4. Human CLDN-3, -4, and -7 were cloned into pCR 3.1 (Invitrogen) and proteins were generated *in vitro* using cDNA clones in the TnT Quick Coupled Transcription/Translation System as determined by Western analysis using antibodies specific for CLDN-3 and -4 (Zymed). Western analysis was performed on equal amounts of protein from TnT reactions using CLDN-7 antibody producing a single band at the predicted size of approximately 23 kDa, but not with antibodies specific to CLDN-3 or -4 (**b**) CLDN-7 protein localizes to the cell membrane, colocalizing with ZO-1. MCF-7 cells were grown to confluence on a chambered slide, and probed with CLDN-7 and ZO-1 antibodies. CLDN-7 and ZO-1 proteins were visualized both individually (red = CLDN-7, green = ZO-1) and as a composite (yellow = colocalization by confocal microscopy at a magnification of $\times 600$

Owing to the heterogeneity of cell types in breast tissue and the fact that only the epithelial cell component expresses CLDNs, it was necessary to determine if the loss of CLDN-7 expression observed in breast cancer tissues relative to normal mammary epithelium was simply because of a difference in epithelial cell content. Therefore, we performed immunohistochemical (IHC) analysis on several of the same IDC cases that had been tested by Western analysis (indicated by * in Figure 3b). In each case, the CLDN-7 staining pattern was compared to that in adjacent normal epithelium as an internal positive control. Surrounding fibroblasts and adipocytes served as negative controls since these cells do not express CLDN proteins. As expected for a tight junction protein, CLDN-7 staining was restricted to epithelial cells with the strongest expression concentrated at the cell membrane (Figure 3c), although diffuse staining in the cytoplasm was also observed. Consistent with the Western analysis results (Figure 3b), the level of CLDN-7 staining was greatly reduced in all three IDCs tested as compared to adjacent normal epithelium (Figure 3c).

Expression of CLDN-7 in ductal carcinoma in situ and IDC

To assess the potential value of loss of CLDN-7 as a prognostic indicator for breast cancer, we determined its expression pattern in a series of *in situ* and invasive breast carcinomas by IHC analysis. As DCIS is believed to be a direct precursor to IDC, we first examined the CLDN-7 staining pattern in a range of DCIS cases, from nuclear grade 1 (low grade) through 3 (high grade). In each case, the staining pattern of CLDN-7 in DCIS was compared to that in adjacent normal epithelium, where staining was predominantly membranous. IHC analysis showed no changes in CLDN-7 expression in either grade 1 (0/10) or grade 2 (0/14) cases, while 71% of grade 3 cases (10/14) showed a loss of its expression (Figure 4, Table 1). Thus, we observed that CLDN-7 expression in

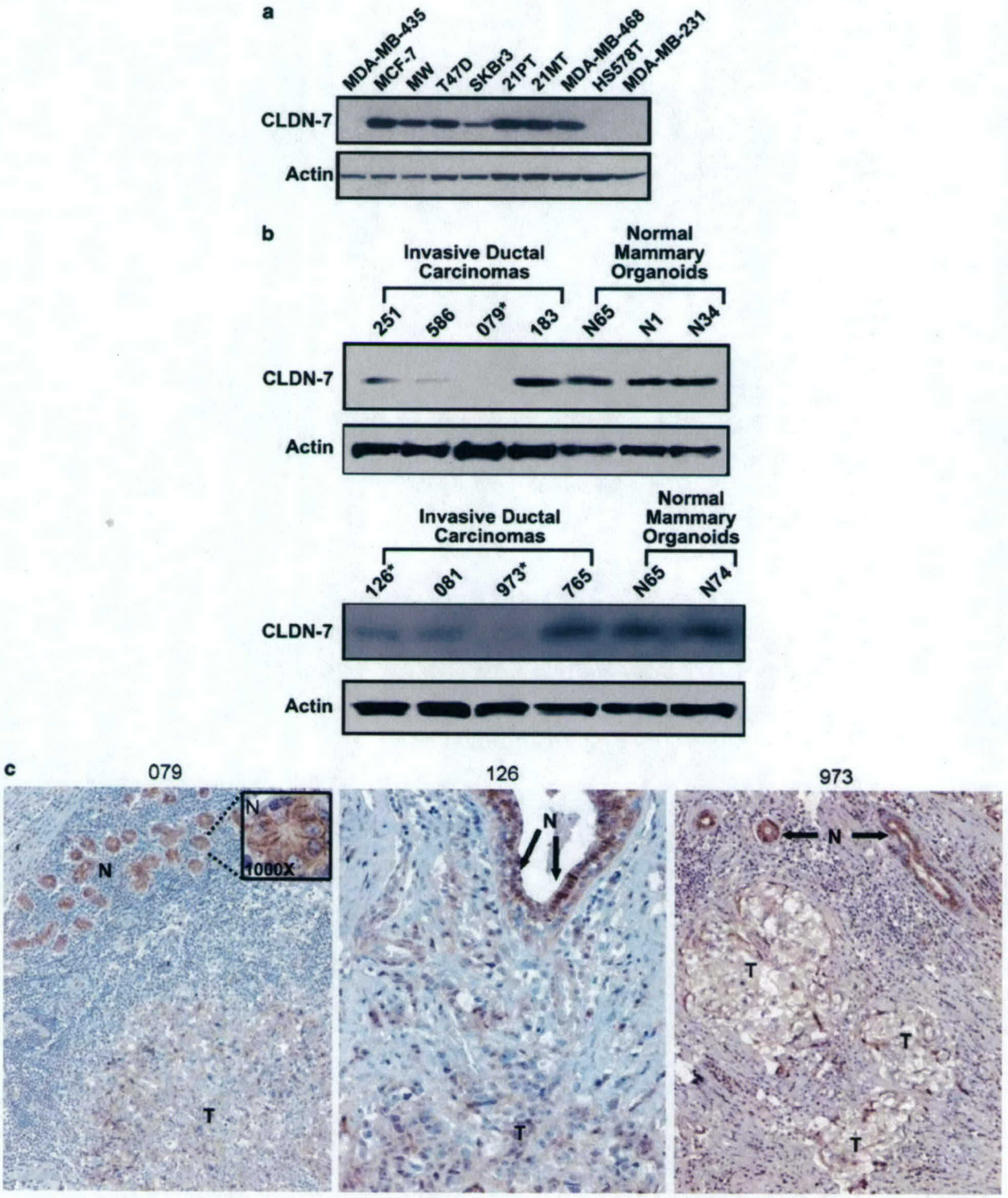

Figure 3 CLDN-7 protein expression in breast cancer cell lines, and IDC relative to normal mammary epithelium. (**a**) CLDN-7 protein expression in breast cancer cell lines. Western analysis was performed on equal amounts of protein from total cell lysates using CLDN-7 and β-actin antibodies. (**b**) IDC and normal mammary organoid tissue were homogenized, and total protein was extracted. Western analysis was performed on equal amounts of protein from cell lysates using CLDN-7 and β-actin antibodies. (**c**) IHC analysis was performed on paraffin-embedded sections of human breast cancer tissues 079, 126, and 973 identified by an asterisk in (**a**) using CLDN-7 antibody. CLDN-7 protein in human breast cancer tissues (T) and adjacent normal mammary epithelium (N) were visualized using DAB. Note the membrane staining in normal breast epithelium (inset). Sections were counterstained with hematoxylin and visualized by light microscopy ($\times$ 200)

DCIS was inversely correlated with nuclear grade ($P<0.001$).

We next examined the CLDN-7 staining pattern in IDCs ranging from Elston grade 1 (low grade) through 3 (high grade), which was compared in each case to that seen in the normal epithelium present on the same section. Few grade 1 (1/6) or grade 2 (3/12) IDC cases showed a loss of CLDN-7 expression, while 77% of grade 3 cases (10/13) showed a significant loss of staining (Figure 4, Table 1). Thus, CLDN-7 expression in IDC was found to be inversely correlated with histological grade ($P=0.014$).

CLDN-7 immunoreactivity in IDC was further studied by tissue array analysis (Figure 5). Of the 612 total cases of IDC on the tissue array, 100 Elston grade 1, 140 Elston grade 2, and 115 Elston grade 3 cases were evaluable and showed an inverse correlation between CLDN-7 expression and histological grade ($P=0.03$). This finding was consistent with the results of the case-by-case analysis

Table 1 IHC analysis of CLDN-7 expression[a]

Histology	*Histological grade*	*Cases with loss of expression/ total cases*	*P*[b]
DCIS	*Nuclear grade*		
	1	0/10	
	2	0/14	
	3	10/14	<0.001
IDC	Elston Grade		
	1	1/6	
	2	3/12	
	3	10/13	0.014
LCIS	NA	13/17	

[a]Data are compiled from IHC analysis of whole paraffin-embedded sections
[b]Comparisons of CLDN-7 expression across grade were made by tabulating scores for CLDN-7 staining according to histological grade (nuclear or Elston grades 1, 2, or 3) Two-sided Fisher's exact tests were used to assess statistical significance

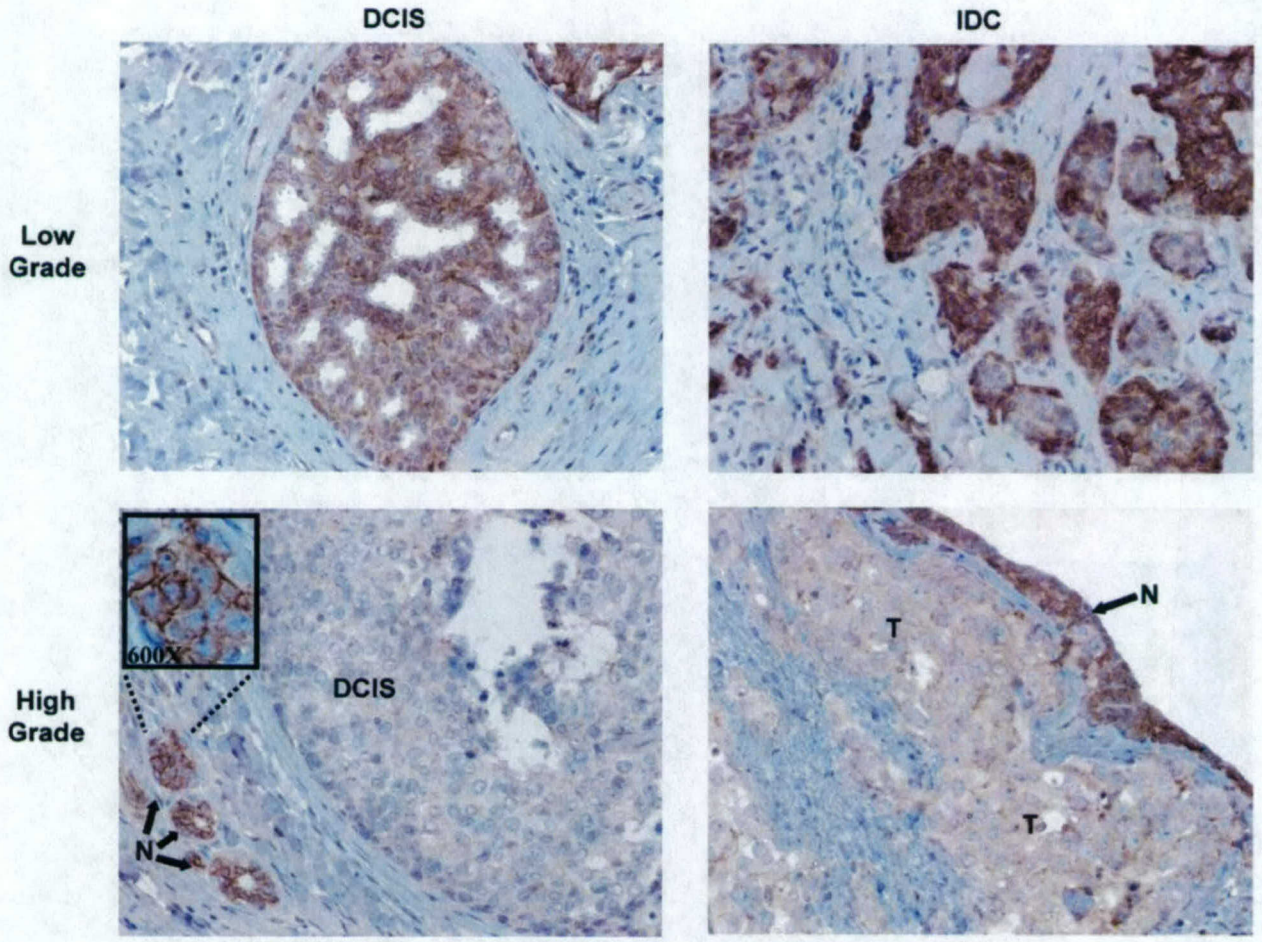

Figure 4 CLDN-7 expression is unchanged in low-grade DCIS (nuclear grades 1–2) and IDC (Elston grades 1–2), but lost in high-grade DCIS (nuclear grade 3) and IDC (Elston grade 3) (T) relative to adjacent normal mammary epithelium (N). IHC analysis was performed on paraffin-embedded sections of human breast tissue using CLDN-7 antibody. CLDN-7 protein was visualized using DAB. Sections were counterstained in hematoxylin and visualized by light microscopy (× 200). Note membrane staining of normal breast epithelium (inset)

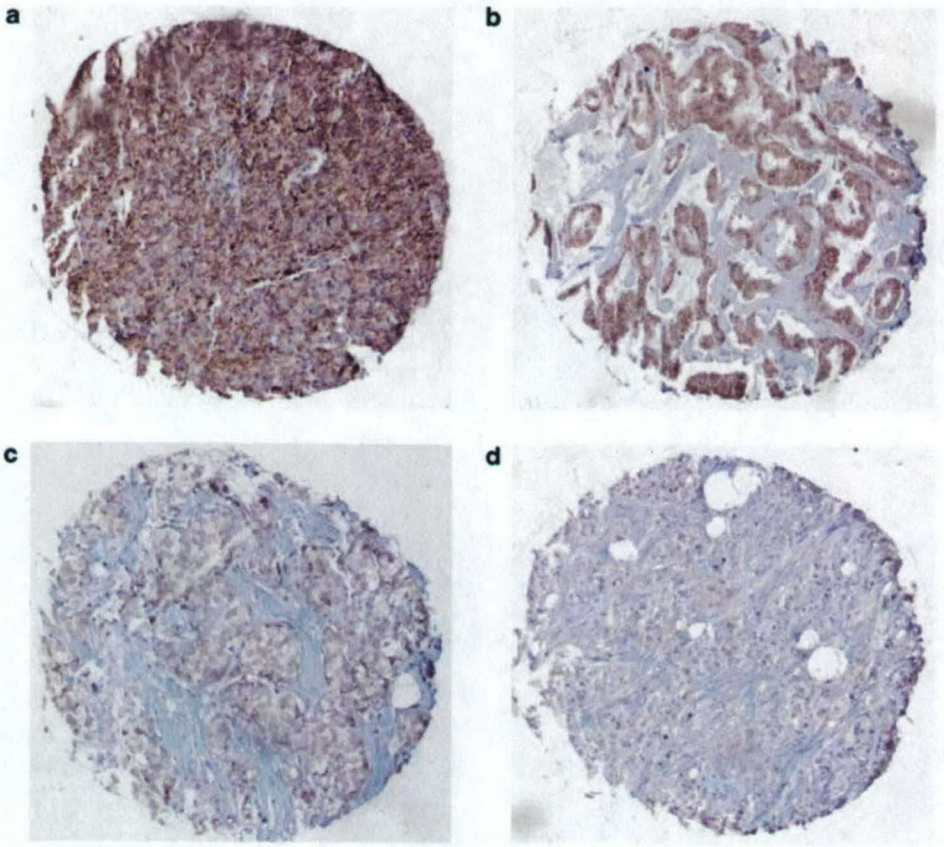

Figure 5 Tissue array analysis of CLDN-7 expression in IDC reveals an inverse correlation between CLDN-7 expression and histological grade. CLDN-7 is expressed in the majority of low-grade IDC (**a, b**), but lost in the majority of high-grade IDC (**c, d**). IHC analysis was performed on tissue arrays containing 612 paraffin-embedded sections of human breast tissue using CLDN-7 antibody. CLDN-7 protein was visualized using DAB. Sections were counterstained in hematoxylin and visualized by light microscopy (× 200)

(summarized in Table 1). No correlation between CLDN-7 expression and estrogen/progesterone receptor status, age, tumor size, or lymph node status was found by tissue array analysis. This last result was contrary to our case-by-case analysis, where seven of 10 IDCs with a positive lymph node status showed a loss of CLDN-7 expression. While the utility of tissue arrays cannot be underestimated since it allows for very high sample throughput, the lack of an internal control (normal epithelium) for each tumor sample, combined with the small sampling represented in each tissue punch could lead to a greater error in determining gene expression. In our study, these factors may be responsible for the lack of correlation with lymph node status in tissue arrays when compared to case-by-case analysis. This source of error is being minimized in newer generations of tissue arrays, which contain several punches from the same tumor tissue, and also from their normal margins. Thus, at the present time, it is critical to perform a case-by-case analysis alongside tissue array analysis.

Expression of CLDN-7 in LCIS

If CLDNs play a functional role in cell-to-cell adhesion, breast lesions that are typified by scattered cells should express very low levels of CLDN-7. In agreement with this notion, IHC analysis of LCIS, a lesion whose defining and characteristic feature is discohesion, showed a loss of CLDN-7 expression in 76% (13/17) of cases. This contrasted significantly ($P = 0.001$) with DCIS, where its loss is seen in only 26% (10/38) of cases irrespective of grade (Figure 6, Table 1).

Effect of HGF/scatter factor on CLDN-7 expression

A direct demonstration of the inverse correlation between CLDN-7 expression and cell-to-cell adhesion was sought by the treatment of breast cancer cell lines with hepatocyte growth factor/scatter factor (HGF/scatter factor). HGF is well known for its ability to decrease cell-to-cell adhesion and stimulate cell migration (Jiang *et al*., 1999). Breast cancer cell lines MCF-7 and T47D, which express high levels of CLDN-7 localized at the tight junction (shown for MCF-7 in Figure 2b), were treated with HGF/scatter factor for a period of 24 h. By Western analysis, a dramatic down-regulation of CLDN-7 was observed in MCF-7, and to a lesser extent in T47D cells (Figure 7). These data provide further direct evidence that loss of CLDN-7 occurs concurrently with loss of cell-to-cell adhesion.

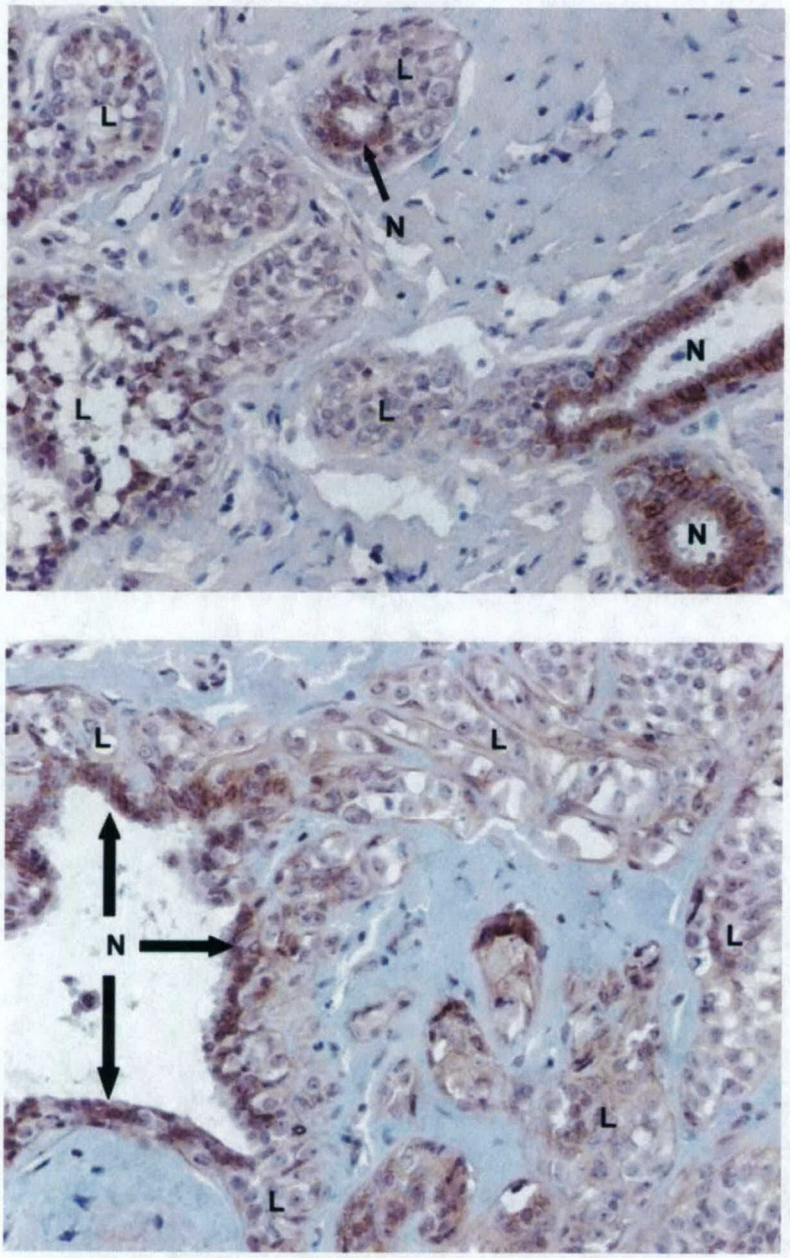

Figure 6 CLDN-7 expression is lost in LCIS (L) relative to adjacent normal mammary epithelium (N). IHC analysis was performed on paraffin-embedded sections of human breast tissue using CLDN-7 antibody. CLDN-7 protein was visualized using DAB. Sections were counterstained in hematoxylin and visualized by light microscopy (× 200)

Mechanism of loss of CLDN-7 expression in breast cancer

To investigate the mechanism responsible for the loss of CLDN-7 expression, we first wanted to rule out the presence of mutations in the CLDN-7 mRNA (Accession #AJ011497) sequence. Nucleotide sequencing of the full-length cDNA revealed no mutations in the CLDN-7 coding sequences in all 11 primary IDCs tested (data not shown). Among the 11 tumors, six expressed very low or no CLDN-7 mRNA as determined by semiquantitative RT–PCR analysis (Figure 1a).

The presence of CG-dinucleotide-rich sequences in the promoter region of genes is quite often a signature denoting that hypermethylation may be a potential mechanism for gene silencing (Ferguson *et al*., 2000; Evron *et al*., 2001a, b; Loeb *et al*., 2001; Jones and Baylin, 2002). The CLDN-7 promoter contains a CpG-rich region extending from −20 to −900 bp upstream of the translational start site (Accession #11425795). Therefore, we investigated the promoter region of the CLDN-7 gene. Methylation-specific PCR (MSP) analysis was performed on DNA from six breast cancer cell lines. The three breast cancer cell lines that show no

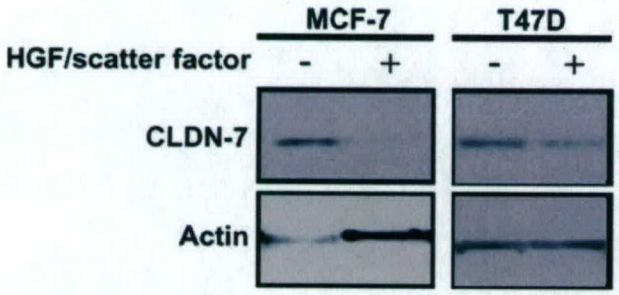

Figure 7 Hepatocyte growth factor/scatter factor (HGF/scatter factor) downregulates CLDN-7 expression in human breast cancer cells. MCF-7 and T47D cells were cultured with or without 10 ng/ml HGF/scatter factor in complete media for 24 h. Western analysis was performed on equal amounts of total cell lysate using CLDN-7 and β-actin antibodies

detectable CLDN-7 expression (HS578T, MDA-MB-231, and MDA-MB-435) contained hypermethylated promoter sequences, while the three that express CLDN-7 (T47D, MCF-7, and MDA-MB-468) were unmethylated in the same region (Figure 8a). This correlation between the loss of CLDN-7 expression and promoter hypermethylation was further confirmed by sequencing a 300 bp region (containing a dense region of 25 CpG dinucleotides, and included the CG-rich region analysed by MSP) of the CLDN-7 promoter, PCR-amplified from sodium-bisulfite-treated DNA. All 25 CpGs were methylated in HS578T, MDA-MB-231, and MDA-MB-435 cells, while MCF-7 cells contained no methylated CpGs (data not shown). Lastly, treatment of HS578T and MDA-MB-435 cells with the demethylating agent, 5-aza-dC, resulted in the re-expression of CLDN-7 (Figure 8b); thus, providing another line of evidence supporting the premise that hypermethylation is a major mechanism responsible for silencing expression of CLDN-7 in breast cancer cell lines.

Next, to determine if hypermethylation-mediated silencing of CLDN-7 expression is functional in primary breast cancer as well, we performed MSP analysis on DNA from IDCs. As expected, the sample of normal mammary organoid, N65, and two IDC samples that express CLDN-7 were unmethylated in this region. However, contrary to our findings in breast cancer cell lines, MSP analysis of the CLDN-7 promoter in the five IDCs that have lost CLDN-7 expression also showed completely unmethylated promoter sequences (Figure 8c). Since MSP analyzes only a few CpGs in the promoter, we sequenced the 300 bp segment of the promoter described above. Sequencing of sodium-bisulfite-treated DNA from IDCs 079 and 973 showed no methylated CpGs (data not shown).

Thus, the evidence provided by MSP, nucleotide sequencing analysis, and re-expression of genes following 5-aza-dC treatment, strongly support the notion that promoter hypermethylation of CLDN-7 is the underlying mechanism for loss of its expression in breast cancer cell lines. However, this is not the case for primary tissue, and alternative mechanisms need to be investigated for loss of CLDN-7 in primary breast tumors.

Discussion

It is widely accepted that the loss of cell-to-cell adhesion is an early event in the process of metastasis, allowing the liberation of individual cancer cells from the primary tumor. Although a functional role for CLDN-7 in breast cancer has not yet been established, there is evidence to support its role in cell-to-cell adhesion, making it plausible that its loss may play a role in metastasis. In this study, we have shown that the level of expression of CLDN-7 mRNA and protein is significantly lower in the majority of breast cancer cell lines and in primary breast carcinomas, than in normal mammary epithelium. By immunohistochemistry, CLDN-7 expression was lost in the majority of high-grade IDCs (Table 1), which are most likely associated with nodal metastasis; this suggests a potential role for CLDN-7 expression in the development of metastasis. Interestingly, in case-by-case analysis, seven of 10 IDCs in our panel that showed a loss of CLDN-7 staining had a positive lymph node status. Although small, these preliminary IHC studies show that CLDN-7 expression in primary IDCs correlates with the ability of the tumor to metastasize. Finally, we showed that hypermethylation of the promoter region correlated with loss of expression of CLDN-7 in breast cancer cell lines, but not in primary breast cancers.

Since high-grade IDCs form fewer tubules and therefore demonstrate less cohesion, it was possible that the expression of all 20 CLDNs would be lost during this transition. However, in several IDC cases in which we observed a loss of CLDN-7 expression, we have found that the levels of CLDN-1, -3, and -4 remain unchanged by both Western and IHC analyses (our unpublished findings). This suggests that the loss of CLDN-7 expression may be a specific event that is not common to all CLDNs. Still the question remains as to whether the loss of one CLDN member could have a significant impact on cell-to-cell adhesion. As numerous CLDNs exist in epithelial tissues, it is reasonable to assume that another CLDN family member may compensate for the loss of CLDN-7. However, a growing body of literature suggests that alterations in individual CLDN proteins can have unique effects in the cell. For example, CLDN-1-deficient mice have been shown to die within 1 day of birth despite the presence of CLDN-4 (Furuse *et al*., 2002). The deletion of CLDN-5 has been found to be associated with velo-cardio-facial syndrome (Sirotkin *et al*., 1997). Mutations in CLDN-14 have been shown to cause autosomal recessive deafness, while mutations in CLDN-16 have been shown to interfere with Mg^{2+} and Ca^{2+} resorption (Simon *et al*., 1999; Wilcox *et al*., 2001). While the 20 known CLDNs may have common and perhaps redundant function in formation of tight junctions, proteins that interact with each CLDN may be unique

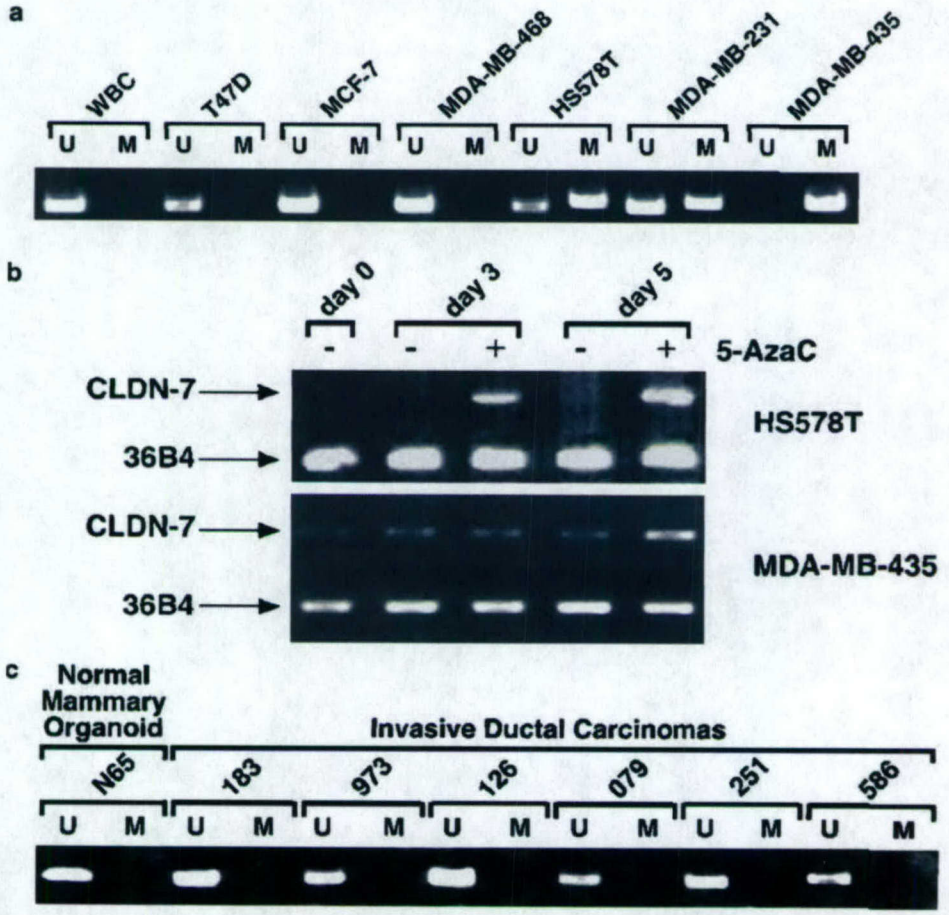

Figure 8 Methylation status of CLDN-7 in breast cancer cell lines and IDC. (**a**) Methylated (M) and unmethylated (U) gene sequences were amplified individually by MSP using sodium-bisulfite-treated DNA from the indicated breast cancer cell lines and WBC: peripheral blood cells (negative control). MSP products were resolved by electrophoresis on a 1.5% agarose gel. (**b**) Re-expression of CLDN-7 in HS578T and MDA-MB-435 cells following treatment with the demethylating agent 5-aza-dC. RT–PCR was performed for CLDN-7 and -36B4, a 'housekeeping' ribosomal protein gene. PCR products were resolved by electrophoresis on a 1.5% agarose gel. (**c**) MSP analysis of sodium-bisulfite-treated DNA from primary invasive ductal carcinomas. N65: normal mammary organoids; 183–586: invasive ductal carcinomas

within specific cell types and thereby confer specificity to the action of each member of this large family of tight junction proteins.

Another important finding of this study is the inverse correlation between CLDN-7 expression and histological grade observed in both DCIS and IDC lesions, which sheds some light on the controversy surrounding the process of breast cancer progression. One possible scenario is that breast cancer progresses in a fashion similar to that of other intraepithelial neoplasias wherein DCIS lesions progress from low-grade to high-grade. High-grade DCIS lesions would then progress to low-grade IDC lesions, which then could progress to high-grade IDCs. The fact that CLDN-7 expression was lost in the majority of high-grade DCIS lesions and high-grade IDC lesions examined, but rarely lost in low-grade IDC lesions (Table 1), does not fit this model. Further, there was no significant difference in the CLDN-7-staining pattern observed between low grades of DCIS and IDC or between high grades of DCIS and IDC (Table 1, Figures 4 and 5). This suggests a model in which low-grade DCIS progresses to low-grade IDC, while high-grade DCIS progresses to high-grade IDC. This model is also supported by the known association of similar histological grades between concurrent DCIS and IDC upon histopathological examination of primary human breast tumors. While the expression pattern of CLDN-7 alone can neither definitively confirm nor deny the validity of either model, it does emphasize the need for further studies into breast cancer progression.

By both MSP and nucleotide sequencing of sodium-bisulfite-treated DNA, we have provided evidence that hypermethylation of promoter sequences is likely the underlying mechanism for the loss of CLDN-7 expression in breast cancer cell lines (Figure 8a, b). In contrast, analysis of primary breast cancer DNA failed to show hypermethylation in the CLDN-7 promoter sequences

(Figure 8c). There is precedence for this observation from our laboratory, where the promoter sequence of WT-1 was hypermethylated and correlated with loss of expression in breast cancer cell lines, but not in primary tumors (Loeb *et al*., 2001). Whether growth in tissue culture requires the loss of expression of certain genes, which could be established by hypermethylation of the promoter, is a valid question. Interestingly, two of the cultured finite lifespan HMEC that lack CLDN-7 mRNA expression, 9F1403 and 184, were hypermethylated in the CLDN-7 promoter region, while two of the uncultured organoid preparations that express CLDN-7 mRNA, N65, and N34 are unmethylated in the same region (our unpublished observations).

In conclusion, this study shows that expression of the tight junction protein CLDN-7 was lost in both preneoplastic and invasive ductal carcinoma of the breast, occurring predominantly in high-grade lesions. CLDN-7 expression was also frequently lost in LCIS, correlating with the increased cellular discohesion observed in LCIS. Additionally, the majority of IDC cases displaying a low CLDN-7 expression had a positive lymph node status. Taken together, these findings suggest that the loss of CLDN-7 may aid in tumor cell dissemination and augment metastatic potential.

Materials and methods

Cell lines, organoids and tumors

Most cell lines were obtained from American Type Culture Collection (Rockville, MD, USA), and cultured according to conditions specified. Breast cancer cell lines 21PT and 21MT were kindly provided by Dr Vimla Band (New England Medical Center, Boston, MA, USA). Finite lifespan HMEC 184, immortalized HMECs 184A1 (early and late passages), and 184B5 were kindly gifted by Dr Martha Stampfer (Lawrence Berkeley Laboratories, Berkeley, CA, USA). Finite lifespan HMECs 9F1403, 04372, 16637 were purchased from Clonetics (Rockville, MD, USA). HMEC strain 11-24 was kindly gifted by Dr Steven Ethier (University of Michigan, Ann Arbor, MI, USA). Mammary organoid samples, N1, N34, N65, and N74, kindly provided by Dr Sigmund A. Weitzman (Northwestern University Medical School, Chicago, IL, USA), were prepared from reduction mammoplasty specimens of women with no abnormalities in the breast as described (Bergstraessar and Weitzman, 1993). Briefly, the specimens were enzymatically digested into duct-like structures (organoids), filtered, histologically confirmed to contain greater than 80% epithelial cells, and frozen at −70°C until use (Bergstraessar and Weitzman, 1993). Highly purified (95–99%) luminal and myoepithelial cells, kindly provided by Dr Lakjaya Buluwela (Division of Medicine, Imperial College School of Medicine, London W120NN, UK), were isolated by differential centrifugation and fluorescence-activated cell sorting of enzymatically digested normal mammoplasty specimens (Gomm *et al*., 1995). Paraffin blocks of DCIS, LCIS, and IDCs of the breast were obtained from the Surgical Pathology files of the Johns Hopkins Hospital, observing institutional guidelines for acquisition of such specimens.

Generation of CLDN-7 antibody

A synthetic peptide corresponding to the C-terminus of CLDN-7 protein conjugated to the carrier protein, keyhole limpet hemocyanin (KLH) was generated by Mimotopes (Raleigh, NC, USA). Polyclonal rabbit antipeptide antibodies were raised and sera were collected. CLDN-7 polyclonal antibody was then affinity purified using the Aminolink Immobilization kit (Pierce, Rockford, IL, USA) and the peptide against which the antibody was raised. To test the affinity-purified CLDN-7 antibody for crossreactivity with other CLDN proteins, human CLDN-3-, -4, and -7 were cloned into pCR 3.1 (Invitrogen, Carlsbad, CA, USA). CLDN-3, -4, and -7 proteins were generated *in vitro* using cDNA clones in the TnT Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA). CLDN-7 antibody is available through Zymed, catalog # 34-9100 h.

Immunofluorescence microscopy

Cells (1×10^5) were plated in eight-chamber slides (Nunc, Naperville, IL, USA) and cultured until confluent. Cells were rinsed in phosphate–buffered saline (PBS) and fixed in 2% paraformaldehyde diluted in PBS for 15 min. Cells were then permeabilized in 0.5% Triton-X diluted in PBS for 5 min. Following permeabilization, cells were incubated in 20 mg/ml bovine serum albumin for 1 h at room temperature. Rabbit polyclonal CLDN-7 antibody diluted at 1 : 500 was then added to the cells and incubated at room temperature for 1 h. Subsequently, cells were incubated with mouse monoclonal ZO-1 antibody (Zymed, San Francisco, CA, USA) for 1 h at room temperature. Cells were then incubated with anti-rabbit IgG conjugated to Alexafluor 568 and anti-mouse IgG conjugated to Alexafluor 488 (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Before visualizing the cells, sections were coverslipped and sealed. Confocal microscopy images were obtained using a Nikon PCM 2000.

Immunohistochemistry

Paraffin-embedded sections and breast tumor array sections were deparaffinized in xylene and rehydrated through graded ethanols. Antigen retrieval was performed by immersing sections in 0.01 M sodium citrate, pH 6.0, and boiling by microwave for 20 min. Sections were then cooled to room temperature and endogenous peroxidase activity was quenched by immersing in 0.3% hydrogen peroxide for 30 min. Blocking was then performed by incubation in diluted normal goat serum (Vectastain kit, Vector, Burlingame, MI, USA) as per the manufacturer's instructions. Sections were then incubated with rabbit polyclonal CLDN-7 at a 1 : 500 dilution for a period of 16 h. Diluted biotinylated anti-rabbit IgG (Vectastain kit) was added to the sections and incubated for 30 min. Vectastain ABC reagent was then added for 30 min. CLDN-7 protein was visualized using 3,3′-diaminobenzamidine (DAB) as per the manufacturer's instructions (Vector). Sections were then counterstained in hematoxylin (Richard-Allan Scientific, Kalamazoo, MI, USA) for 10 s. Lastly, sections were dehydrated through graded ethanols, cleared in xylene, mounted, and coverslipped. Images were acquired by light microscopy.

Statistical analysis of CLDN-7 expression

IHC staining of CLDN-7 in DCIS, LCIS, and IDC lesions was scored relative to adjacent normal mammary epithelium as positive (no change in expression) or negative (loss of expression). Comparisons of CLDN-7 expression across grade were made by tabulating scores for CLDN-7 staining according to histological grade (Nuclear or Elston grades 1, 2, or 3). Two-sided Fisher's exact tests were used to assess

statistical significance. Although grade is an ordinal variable, the analysis treated it as nominal categorical. As such, *P* values are slightly conservative. Inverse correlation implies that as histological grade tends to higher values, CLDN-7 is less likely to be expressed.

Methylation-specific PCR

Genomic DNA (1 μg) was treated with sodium bisulfite as previously described (Ferguson *et al.*, 2000) and was analysed by MSP using primer sets located within a CpG-rich area in the CLDN-7 promoter (Accession #11425795). Primers specific for unmethylated DNA were 5′-TGGGGAAAGGG TGGTGTTG-3′ (sense, −831 to −812) and 5′-TTACC-CAATTTTAACCACCAC-3′ (antisense, −670 to −649) yielding a 182 bp product. Primers specific for methylated DNA were 5′-GACGTTAGGTTATTTTCGGTC-3′ (sense, −550 to −529) and 5′-AAACGCGTTTCTAAACGCCG-3′ (antisense, −350 to −330) yielding a 220 bp product. The PCR conditions were as follows: one cycle of 95°C for 5 min 'hot start,' then addition of 1 μl Taq polymerase (RedTaq, Sigma, St Louis, MO, USA); 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s; and one cycle of 72°C for 5 min. PCR samples were resolved by electrophoresis on a 1.5% agarose gel.

5-aza-dC treatment

Cells were seeded in a 100 mm plate at a density of 1×10^6 cells. After 24 h, cells were treated with 0.75 μM 5-aza-dC (Sigma) (Ferguson *et al.*, 2000; Evron *et al.*, 2001a, b). Total cellular DNA and RNA were isolated at 0, 3, and 5 days after addition of 5-aza-dC.

RT–PCR

Total RNA was extracted using TRI REAGENT BD by the manufacturer's protocol (Molecular Research Center, Cincinnati, OH, USA). cDNA was generated by reverse transcription. Reactions contained 2 μg DNAse-treated RNA, 0.25 μg/ul pdN6 random primers (Life Technologies, Rockville, MD, USA), 1 × first-strand buffer (Life Technologies), 1 mM of each deoxynucleotide triphosphate (Life Technologies), 200 units Superscript reverse transcriptase (Life Technologies), and were incubated for 1 h at 37°C, followed by heat inactivation at 70°C for 15 min. PCR was performed using the primers 5′-CCACTCGAGCCCTAATGGTG-3′ (sense) and 5′-GGTA CCCAGCCTTGCTCTCA-3′ (anti-sense) for CLDN-7 (Accession #AJ011497). Coamplified products of 36B4, a 'housekeeping' ribosomal protein gene, were used as an internal control, using primers 5′ GATTGGCTACCCAACTGTTG-CA-3′ and 5′-CAGGGGCAGCAGCCACAAAGGC-3′ for sense and antisense, respectively. The 25 μl reactions contained 1X buffer (2X reaction mix, Life Technologies), 1 μl cDNA, and 100 nm of each primer. The PCR conditions were: one cycle of 94°C for 1 min, 'hot start,' followed by addition of one unit of Taq polymerase (RedTaq, Sigma), 35 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 45 s, and finally one cycle of 72°C for 5 min. PCR samples were resolved by electrophoresis on a 1.5% agarose gel.

Real–time PCR

Total RNA was extracted and cDNA was generated by reverse transcription as described above. CLDN-7 and GAPDH (a 'housekeeping' gene) were amplified individually using a 96-well plate and optical caps (PE Applied Biosystems, Foster City, CA, USA) with a 25 μl final reaction volume containing 250 nmol/l sense and antisense primer, 200 nmol/l probe, 2.5 mM $MgCl_2$, one unit Amplitaq Gold, 200 μmol/l each of dATP, dCTP, dTTP, and dGTP in 1X Taqman Buffer A. Reaction mixtures were preheated to 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The primer and probe sequences are as follows: CLDN-7 (sense) 5′-AAAG TGAAGAAGGCCCGTATAGC-3′, CLDN-7 (antisense) 5′-GCTACCAAGGCGGCAAGAC-3′, CLDN-7 (probe) 5′-CC ACGATGAAAATTATGCCTCCACCCA-3′, GAPDH (sense) 5′-CCCATGTTCGTCATGGGTGT-3′, GAPDH (antisense) 5′-TGGTCATGAGTCCTTCCACGATA-3′, and GAPDH (probe) 5′-CTGCACCACCAACTGCTTAG-3′. All PCR reagents, including primers and probes, were purchased from PE Applied Biosystems.

Sequencing of sodium-bisulfite-treated DNA

DNA from peripheral white blood cells, IDCs, and breast cancer cell lines was treated with sodium bisulfite as previously described (Ferguson *et al.*, 2000). Briefly, the DNA was purified and a CpG-rich promoter region was amplified by PCR using the following primers: 5′-GTGATTTTGGTGTT-TAGGT-3′ (sense primer with start at −675) and 5′-ATCCCAAAATATCCTAAACTA-3′ (antisense primer with start at −375), which generated a 300 bp PCR product. The product was purified using a Qiagen PCR purification kit (Qiagen Corp) and sequenced using the antisense primer.

Western blotting

IDC of the breast and normal mammary organoid tissue was homogenized and total protein was extracted using lysis buffer consisting of 15% glycerol, 5% SDS, and 250 mM Tris-HCl, pH 6.7. Equal amounts of protein from cell lysates were resolved using 12% SDS-PAGE (Invitrogen, Carlsbad, CA, USA). Protein was then transferred to ECL nitrocellulose membranes (Amersham, Arlington Heights, IL, USA). Following Western transfer, membranes were probed with CLDN-3, 4 (Zymed), CLDN-7, or β-actin (Amersham) antibody diluted 1 : 1000 (CLDN-3, 4, and 7) or 1 : 5000 (β-actin). Horseradish peroxidase-conjugated antibody against rabbit or mouse IgG (Amersham) was used at 1 : 1000 and binding was revealed using enhanced chemiluminescence (Amersham).

Abbreviations

CLDN, Claudin; IDC, invasive ductal carcinoma; RT–PCR, reverse transcription–PCR; IHC, immunohistochemical analysis; DCIS, ductal carcinoma *in situ*; LCIS, lobular carcinoma *in situ*; HGF/scatter factor, hepatocyte growth factor/scatter factor; KLH, keyhole limpet hemocyanin; MSP, methylation-specific PCR; DAB, 3 3′-diaminobenzamidine; GAPDH, glyceraldehyde phosphate dehydrogenase; 5-aza-dC, 5′-aza-2′-deoxycytidine; HMEC, human mammary epithelial cells.

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Clostridium perfringens Enterotoxin Elicits Rapid and Specific Cytolysis of Breast Carcinoma Cells Mediated through Tight Junction Proteins Claudin 3 and 4

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From the Breast Cancer Program, Sidney Kimmel Comprehensive Cancer Center and the Department of Pathology,§ Johns Hopkins University School of Medicine, Baltimore, Maryland; the Biomedical Research Institute,† North Shore-Long Island Jewish Health System, Manhasset, New York; and Northwestern University Medical School,‡ Chicago, Illinois*

Clostridium perfringens* enterotoxin (CPE) induces cytolysis very rapidly through binding to its receptors, the tight junction proteins CLDN 3 and 4. In this study, we investigated CLDN 3 and 4 expression in breast cancer and tested the potential of CPE-mediated therapy. CLDN 3 and 4 proteins were detected in all primary breast carcinomas tested (n = 21) and, compared to normal mammary epithelium, were overexpressed in approximately 62% and 26%, respectively. Treatment of breast cancer cell lines in culture with CPE resulted in rapid and dose-dependent cytolysis exclusively in cells that expressed CLDN 3 and 4. Intratumoral CPE treatment of xenografts of T47D breast cancer cells in immunodeficient mice resulted in a significant reduction in tumor volume (P = 0.007), with accompanying necrosis. Necrotic reactions were also seen in three freshly resected primary breast carcinoma samples treated with CPE for 12 hours, while isolated primary breast carcinoma cells underwent rapid and complete cytolysis within 1 hour. Thus, expression of CLDN 3 and 4 sensitizes primary breast carcinomas to CPE-mediated cytolysis and emphasizes the potential of CPE in breast cancer therapy. *(Am J Pathol 2004, 164:1627–1633)

Breast cancer is the second leading cause of cancer death in women. While medical advances have significantly improved long-term survival of women diagnosed at the early stages, this has not been true for women with advanced breast cancer. Thus, innovative therapeutic modalities are sorely needed. The ability of *Clostridium perfringens* enterotoxin (CPE) to directly and rapidly lyse mammalian cells has been known for over 20 years[1] and is responsible for the gastrointestinal symptoms associated with *C. perfringens* type A food poisoning.[2] These symptoms are elicited following the release of CPE into the intestinal lumen, where it binds to its receptors on the surface of intestinal epithelial cells. This triggers the formation of a large multiprotein membrane pore complex and ultimately results in cell lysis.[3] CPE-mediated cytolysis has been shown to occur extremely rapidly, requiring only 5 to 15 minutes.[4] The process is very specific, since cells lacking expression of CPE receptors are completely unaffected by the toxin.[5] Recently, Claudins (CLDN) 3 and 4 were identified as the receptors for CPE.[6,7] The CLDN family of proteins, which function in the sealing of tight junctions, was discovered in 1998.[8] Although more than 18 CLDN proteins have been identified, only CLDN 3 and 4 were found to sensitize cells to CPE-mediated cytolysis.[9,10] Interestingly, CLDN 3 and 4 are highly expressed in several cancer types.[11–13] The ability of CPE to rapidly and specifically lyses cells expressing CLDN 3 and/or 4, raised the possibility that the toxin may be useful in the treatment of these cancers.[11–13] Here we show that CLDN 3 and 4 are consistently expressed in primary breast carcinomas and breast cancer cell lines. Additionally, CLDN 3 and 4 are overexpressed in approximately 62% and 26% of primary breast carcinomas, respectively, relative to normal mammary epithelium. The expression of CLDN 3 and 4 sensitizes them to CPE-mediated cytolysis, suggesting that this potently cytotoxic enterotoxin, when delivered locally, may be very useful in breast cancer therapy.

Materials and Methods

Cell Lines, Organoids, and Tumors

Most cell lines were obtained from American Type Culture Collection (Rockville, MD), and cultured according to conditions specified. Breast cancer cell lines 21PT and 21MT were kindly provided by Dr. Vimla Band (New England Medical Center, Boston, MA). Finite lifespan human mammary epithelial cells (HMEC) 286, 6–8, 11–

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24, 9–10, and 5–24 were gifts from Dr. Steven Ethier (University of Michigan, Ann Arbor. MI). Mammary epithelial organoid samples (N74, B31, and B54) were prepared from reduction mammoplasty specimens of normal women as described.[14] Briefly, the specimens were enzymatically digested into duct-like structures (organoids), filtered, histologically confirmed to contain greater than 70% epithelial cells, and frozen at −70°C until use. Freshly resected primary breast carcinoma samples and paraffin blocks of primary breast carcinoma tissue were obtained from the Surgical Pathology Division of the Johns Hopkins Hospital, observing institutional guidelines for acquisition of such specimens.

Western Blotting

Primary breast carcinoma tissue containing greater than 70% epithelial cells as determined by H&E staining and normal mammary organoid tissue were homogenized. Total protein was extracted from homogenized breast tissues, HMEC, and breast cancer cell lines using lysis buffer consisting of 15% glycerol, 5% SDS, and 250 mmol/L Tris-HCl, pH 6.7. Equal amounts of protein from cell lysates were resolved using 12% SDS-PAGE (Invitrogen, Carlsbad, CA). Protein was transferred to ECL nitrocellulose membranes (Amersham, Arlington Heights, IL). Following Western transfer, membranes were probed with anti-human CLDN 3 (Zymed, San Francisco, CA), anti-human CLDN 4 (Zymed), or β-actin (Amersham) antibody diluted 1:1000 (CLDN 3 and 4) or 1:5000 (β-actin). Horseradish peroxidase-conjugated antibody against rabbit or mouse IgG (Amersham) was used at 1:1000 and binding was revealed using enhanced chemiluminescence (Amersham).

Immunohistochemistry

Paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded ethanols. Antigen retrieval was performed by immersing sections in 0.01 mol/L sodium citrate, pH 6.0, and boiling by microwave for 20 minutes. Sections were then cooled to room temperature and endogenous peroxidase activity was quenched by immersing in 0.3% hydrogen peroxide for 30 minutes. Blocking was then performed by incubation in diluted normal goat (CLDN 3) or horse (CLDN 4) serum (Vectastain kit, Vector, Burlingame, MI) as per the manufacturer's instructions. Sections were then incubated with rabbit polyclonal CLDN 3 or mouse monoclonal CLDN 4 at a 1:500 dilution for a period of 16 hours. Diluted biotinylated anti-rabbit or anti-mouse IgG (Vectastain kit) was added to the sections and incubated for 30 minutes. Vectastain ABC reagent was then added for 30 minutes. CLDN 3 and 4 protein was visualized using 3,3′-diaminobenzamidine (DAB) as per the manufacturer's instructions (Vector). Sections were then counterstained in hematoxylin (Richard-Allan Scientific, Kalamazoo, MI) for 10 seconds. Lastly, sections were dehydrated through graded ethanols, cleared in xylene, mounted, and coverslipped. Images were acquired by light microscopy.

Cytotoxicity Assays

CPE was isolated and purified as previously described.[13] The breast cancer cell lines MCF-7, SKBr3, T47D, HS578T, and MDA-MB-435 were plated in 6-well plates and grown to approximately 80% confluence in complete medium. Old medium was then removed and replaced with complete medium with or without CPE at concentrations ranging from 0.05 to 4 μg/ml. Cells were then incubated at 37°C for 60 minutes. Floating cells were collected and pooled with adherent cells removed by trypsinization. Total cells were then counted using a hemacytometer, and cell viability was determined by trypan blue dye (0.4%) exclusion.

Freshly resected primary breast tumor samples of approximately 5 to 10 mm^3 were divided into two equal pieces and incubated with or without 10 μg of CPE in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) for a period of 12 hours. Tissues were subsequently fixed in 10% neutral buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin.

A surgically resected high grade (Elston grade III) primary breast carcinoma was rapidly cut into small pieces under sterile conditions, placed in DMEM, and digested with collagenase A (Boehringer Mannheim, Mannheim, Germany) for a period of 2 hours at 37°C, rotated at 225 rpm. Digested tumor tissue was then filtered sequentially through 250-, 100-, and 40-μm filters creating a single cell suspension. Primary breast carcinoma cells were then isolated by incubation with the epithelia-specific antibody BerEP4 conjugated to magnetic beads (Dynal Biotech ASA, Oslo, Norway). Beads were washed 10 times in phosphate buffered saline with 0.5% bovine serum albumin (PBS/BSA). Primary breast carcinoma cells and attached beads were resuspended in PBS/BSA, divided equally into a 6-well plate, and incubated with or without 2 μg/ml CPE in DMEM supplemented with 10% FBS for a period of 60 minutes at 37°C. Percent cytotoxicity was determined by comparison of non-ballooned cell counts within a demarcated 1-cm^2 grid at 0 and 60 minutes following the addition of CPE as previously described.[6]

Xenografts in Mice

SCID mice were kindly provided by Dr. Curt Civin (Johns Hopkins University School of Medicine, Baltimore, MD) and animals were maintained in a pathogen-free environment. Xenografts were generated in 6- to 8-week-old animals. Two days before injection of tumor cells, animals received one intramuscular injection of 1.5 mg/kg depoestradiol (Florida Infusion Co., Palm Harbor, FL) diluted 1:3 in cottonseed oil. T47D human breast cancer cells were washed twice in serum-free DMEM and resuspended in Matrigel (BD, Franklin Lakes, NJ) at a concentration of 1×10^7 cells/0.1 ml. T47D cells (1×10^7) were injected subcutaneously in the left and right flank. Animals received an intramuscular injection of 0.5 mg/kg

depo-estradiol diluted 1:3 in cottonseed oil at 1 and 2 weeks following initial estradiol administration. Tumor sizes were determined by measuring three diameters using calipers. On reaching a size of approximately 100 mm^3, tumors were administered intratumoral injections of 2 μg of CPE, 10 μg of CPE, or PBS on days 1, 3, 5, 7, 9, 11, and 13. Tumor size was recorded on days 1, 7, and 14, after which animals were euthanized and tumors were removed. Tumors were fixed in 10% neutral buffered formalin for histological examination by hematoxylin and eosin (H&E) and Ki67 staining. Each experiment consisted of six treated tumors and six control tumors.

Results

Expression of CPE Receptors, CLDN 3 and 4, in Breast Cancer Cell Lines, Primary Breast Carcinoma, and Normal Mammary Epithelium

Recently, CLDN 3 and 4 were reported to be overexpressed in both ovarian and pancreatic carcinomas relative to normal epithelium as determined by serial analysis of gene expression (SAGE).[11,15] Similarly, using SAGE analysis we found CLDN 3 and 4 mRNA to be overexpressed by two- to threefold in several breast cancer cell lines relative to finite life-span human mammary epithelial cells (HMEC).[16] To validate these findings we performed Western blot analysis using a panel of breast cancer cell lines, primary breast carcinomas, and normal human mammary epithelial cells. CLDN 3 and 4 were consistently expressed in all primary breast carcinomas tested (15/15) and in 60% (CLDN 3) and 80% (CLDN 4) of breast cancer cell lines (n = 10) (Figure 1, A and B). In agreement with the results of our SAGE analysis,[16] CLDN 3 and 4 proteins were overexpressed by more than twofold in 12/15 (P = 0.008) and 5/15 (P = 0.046) primary breast carcinomas, respectively, relative to HMEC and normal epithelial organoids obtained from reduction mammoplasty specimens as determined by densitometric scanning (Figure 1C).

Although both the primary breast carcinoma and normal mammary organoid samples used were verified to contain greater than 70% epithelial cells, it is possible that the increase in CLDN 3 and 4 expression observed in primary breast carcinomas relative to normal mammary organoids was influenced by differences in the epithelial cell content of the tissue, since CLDN 3 and 4 are solely expressed by the epithelial cell component of human breast tissue. To address this possibility, we performed immunohistochemical (IHC) analysis of 10 primary breast carcinoma cases of varying histological grade, 4 of which were included in our Western blot analysis (identified by asterisk in Figure 1A). In each case the CLDN 3 and 4 staining patterns were compared to that in adjacent normal epithelium. Surrounding fibroblasts and adipocytes served as negative controls since these cells do not express CLDN proteins. Consistent with our Western blot analysis, CLDN 3 and 4 staining was detectable in all primary breast carcinomas tested and was higher in 5/10 and 3/10 primary breast carcinomas, respectively, compared to levels present in adjacent normal mammary epithelium (Figure 1D). In agreement with previous findings, CLDN 3 and 4 expression was restricted to epithelial cells and localized to the cell membrane, consistent with the biological role of CLDNs in the formation of tight junctions.

Efficacy of CPE-Mediated Cytolysis on Breast Cancer Cell Lines in Vitro

Although the cytolytic ability of CPE has been demonstrated in various cell types,[4,12,13] the effect of CPE on breast cancer cells has not been characterized. To determine the effect of CPE on breast cancer cells, we treated three breast cancer cell lines that show expression of CLDN 3 and 4 (MCF-7, SKBr3, and T47D) and two breast cancer cell lines lacking detectable expression of CLDN 3 and 4 (HS578T and MDA-MB-435) with concentrations of CPE ranging from 0.05 to 4 μg/ml for a period of 60 minutes. Following CPE treatment, all of the cells in the culture dish were counted and percent cytotoxicity was determined by trypan blue dye exclusion. CPE treatment of breast cancer cell lines expressing CLDN 3 and 4 resulted in rapid and virtually complete cytolysis in a dose-dependent fashion (Figure 2A). Consistent with previous studies in cell lines from other carcinomas, breast cancer cell lines lacking CLDN 3 and 4 expression were completely resistant to the cytotoxic effects of CPE (Figure 2B). Thus, CPE treatment results in rapid and potent cytolysis specific to breast cancer cell lines expressing CLDN 3 and 4.

Efficacy of CPE-Mediated Cytolysis of Breast Cancer Cell Line T47D in Vivo

Next, we investigated whether CPE has cytolytic effects on breast cancer cells grown as tumors *in vivo*. Xenograft tumors were grown subcutaneously in SCID mice using the CLDN 3 and 4-expressing breast cancer cell line, T47D. Tumors were allowed to grow to a size of approximately 100 mm^3 and then treated with a total of seven intratumoral injections of either 2 μg of CPE, 10 μg of CPE, or PBS alone administered every other day over the course of 14 days. Tumor volumes were measured on days 1, 7, and 14, after which all animals were euthanized and tumors were removed for histological examination. Animals receiving CPE did not exhibit any signs of systemic toxicity such as loss of weight, huddling, ruffled fur, etc. The effect of CPE on T47D xenograft tumors was dose-dependent. Tumors receiving intratumoral injections of 2 μg of CPE showed no increase in tumor volume between days 1 and 14, while the volume of tumors receiving 10 μg of CPE per injection was significantly reduced at day 14 relative to the volume on day 1 (P = 0.007) (Figure 3, A and B). Further, on histological examination, tumors treated with either dose of CPE showed large areas of necrosis as evidenced by H&E staining of tumor sections (Figure 3C). The extent of cell death was confirmed by staining for the proliferation marker Ki67, which showed the presence of very few viable tumor cells following CPE treatment. This observation directly corre-

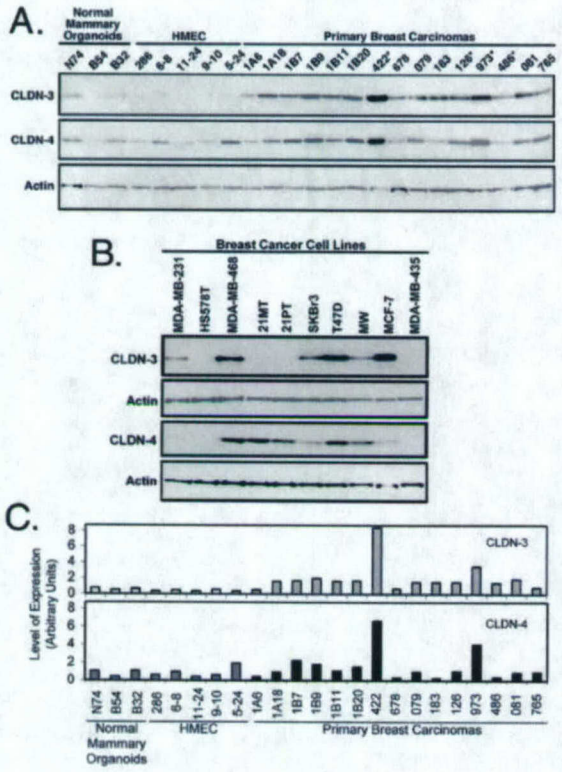
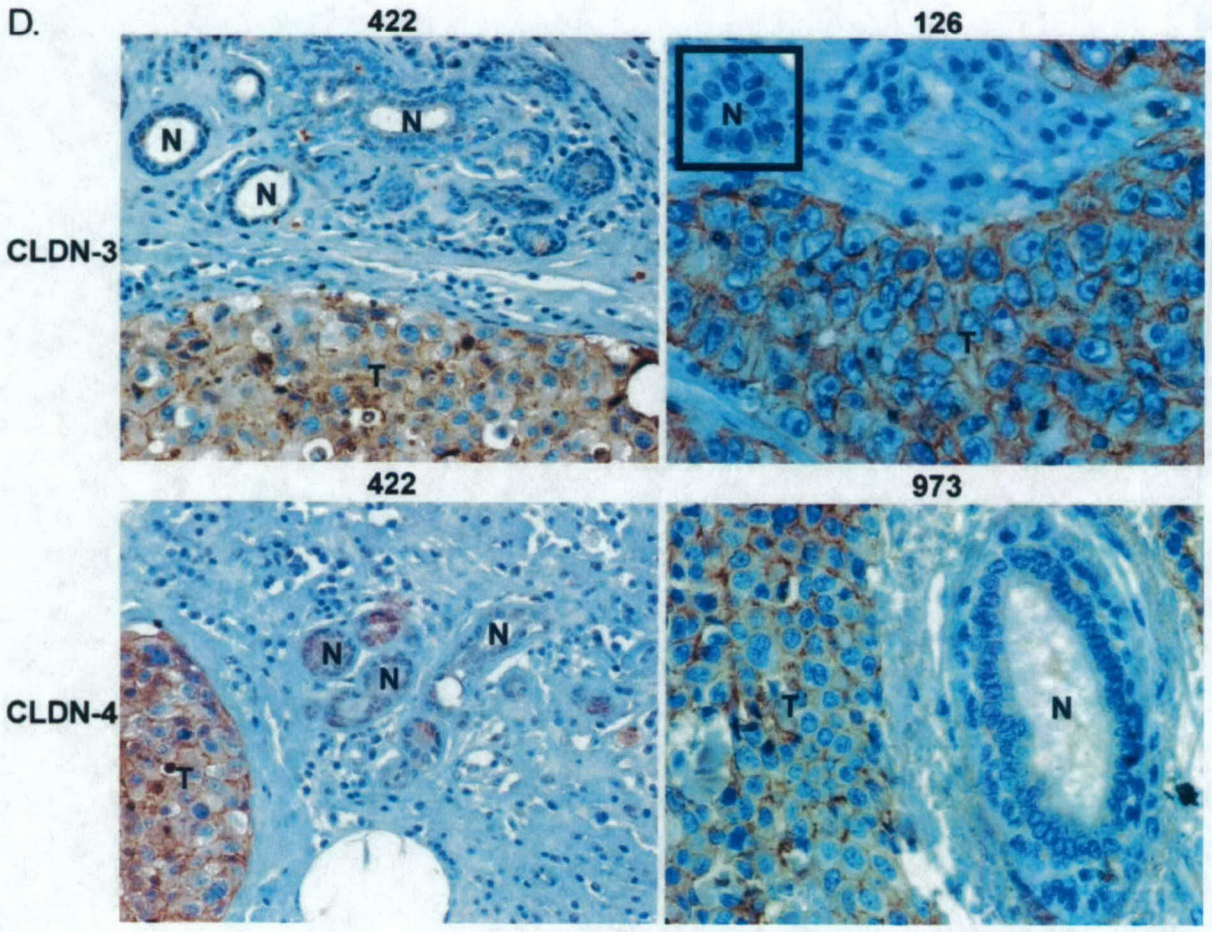


Figure 1. Expression of CLDN 3 and 4 proteins in breast cancer cell lines, primary breast carcinoma, and normal mammary epithelium. **A:** Human primary breast carcinoma, normal mammary organoid tissue, and finite life span human mammary epithelial cells (HMEC) were homogenized and total protein was extracted. Western analysis was performed on equal amounts of protein from cell lysates using CLDN 3, 4, and β-actin antibodies. Significant differences in CLDN 3 ($P = 0.008$) and CLDN 4 ($P = 0.046$) expression levels between primary breast carcinomas and normal mammary epithelial cells were determined by Student's *t*-test. **B:** CLDN 3 and 4 protein expression in human breast cancer cell lines. Western analysis was performed on equal amounts of protein from total cell lysates using CLDN 3, 4, and β-actin antibodies. **C:** The level of CLDN 3 and 4 expression in primary breast carcinomas, normal mammary organoids, and HMEC normalized to actin were determined by densitometric scanning of radiographical film. **D:** Immunohistochemical analysis was performed on paraffin embedded sections of human primary breast carcinoma tissues identified by an **asterisk** in **A** using CLDN 3 and 4 antibodies. CLDN 3 and 4 proteins in primary breast carcinoma tissue and adjacent normal mammary epithelium were visualized using DAB. Sections were counterstained with hematoxylin and visualized by light microscopy (422, ×200; 126 and 973, ×400).



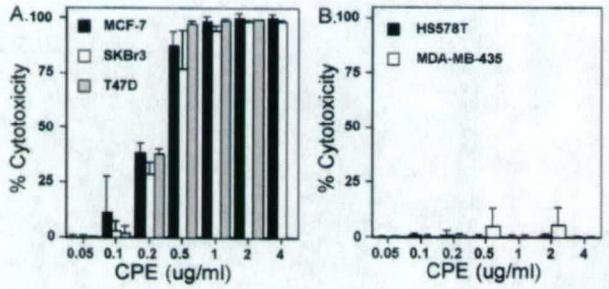


Figure 2. Sensitivity of human breast cancer cell lines to CPE-mediated cytolysis *in vitro*. CLDN 3 and 4 expressing (MCF-7, SKBr3, and T47D; **A**) and non-expressing (HS578T and MDA-MB-435; **B**) breast cancer cell lines were incubated in complete media with or without CPE at concentrations ranging from 0.05 to 4 μg/ml for 60 minutes. Total cells were pooled and counted using a hemacytometer and cell viability was determined by trypan blue dye (0.4%) exclusion. Data from representative experiments performed in triplicate are expressed as % cytotoxicity ±SD.

lates with the effects of CPE on tumor volume (Figure 3C). These experiments show that CPE treatment of T47D xenograft tumors *in vivo* results in cell necrosis, with a significant reduction in tumor volume.

Susceptibility of Primary Breast Carcinoma to CPE-Mediated Cytolysis

Although we have shown expression of CLDN 3 and 4 in all primary breast carcinomas tested (Figure 1A), the susceptibility of primary tumor tissue to CPE-mediated

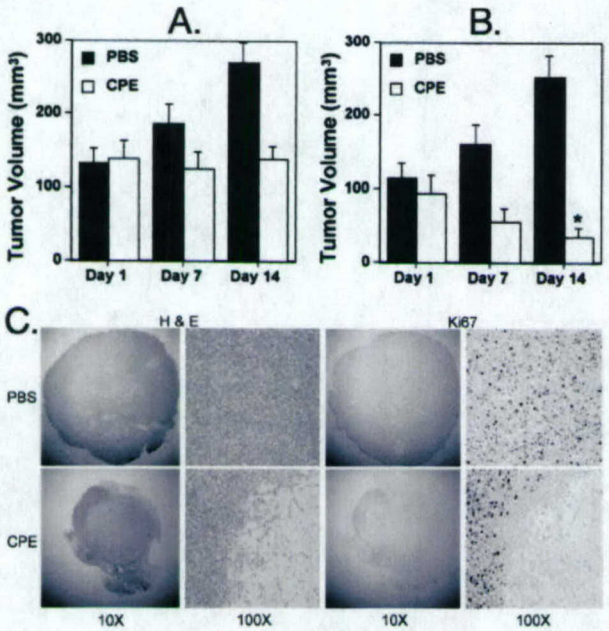


Figure 3. Sensitivity of human breast cancer cell line T47D to CPE-mediated cytolysis *in vivo*. Xenograft tumors were established in SCID mice using the human breast cancer cell line T47D. Tumors were allowed to grow to a size of approximately 100 mm^3 and subsequently treated by intratumoral injection of 2 μg of CPE (**A**) or 10 μg of CPE (**B**) versus PBS alone on days 1, 3, 5, 7, 9, 11, and 13. Tumor volumes were measured on days 1, 7, and 14, after which animals were euthanized, tumors were removed, and tumor viability was determined by H&E and Ki67 staining (**C**). Each experiment is representative of 12 tumors. $^*P = 0.007$ compared to volume on day 1 as determined by Student's *t*-test.

cytolysis remained to be tested. We subjected three freshly resected breast tumor tissues of various histological grades to CPE treatment *in vitro*. Tumor tissue samples of approximately 5 to 10 mm^3 were divided into two equal pieces and incubated in complete media with or without 10 μg of CPE for a period of 12 hours to allow diffusion of CPE into the tissue. Tissues were subsequently fixed in 10% neutral buffered formalin, paraffin embedded, and sectioned. Subsequent histopathological analysis of H&E stained tumor sections revealed a consistently higher degree of tumor cell necrosis in tissues treated with CPE as compared to media alone (Figure 4A). No effect was seen on vascular endothelial or stromal cells, consistent with the absence of CLDN 3 and 4 expression in these cell types.

The susceptibility of primary breast carcinoma to CPE-mediated cytolysis was further explored by isolating carcinoma cells from a freshly resected Elston grade III breast tumor by immunopurification. One half of the carcinoma cells were treated with CPE (2 μg/ml) for 60 minutes, while the second half was placed in media alone. Percent cytotoxicity was then determined by non-ballooned cell counts as described previously.[6] The breast cancer cell lines MCF-7 and HS578T were included as positive and negative controls, respectively. CPE treatment of epithelial cells isolated from the tumor resulted in nearly 100% cytotoxicity. This cytotoxic response was equivalent to the effect of CPE on CLDN 3 and 4-positive MCF-7 cells (Figure 4B). Consistent with previous results, no loss of viability was observed in HS578T cells that lack detectable CLDN 3 and 4 expression. These results indicate that primary breast cancer cells are also susceptible to the cytolytic effects of CPE, a response that is likely mediated through the binding of CPE to its receptors, CLDN 3 and 4, expressed at the cell membrane.

Discussion

CPE is a well-known virulence factor responsible for the gastrointestinal symptoms associated with *C. perfringens* type A food poisoning. However, its ability to rapidly and specifically lyse cells expressing its receptors, CLDN 3 and 4, could be effectively exploited in the treatment of cancers constitutively expressing these proteins. In this study we have shown overexpression of both CLDN 3 and 4 in approximately 62% and 26% of primary breast carcinomas, respectively, relative to normal mammary epithelium. CLDN 3 and 4-expressing breast cancer cell lines grown in cell culture and as xenograft tumors underwent rapid and dose-dependent cytolysis in response to CPE treatment. Consistent with our observations in cultured breast cancer cell lines, primary breast tumor samples as well as isolated carcinoma cells from freshly resected tumor specimens also underwent CPE-mediated cytolysis *in vitro*. These results raise the possibility that CPE treatment *in vivo* may be an effective targeted therapy for breast cancer.

Despite the ability of CPE to effectively lyse breast cancer cells, its clinical application faces obstacles similar to those of other protein-based therapeutics. A com-

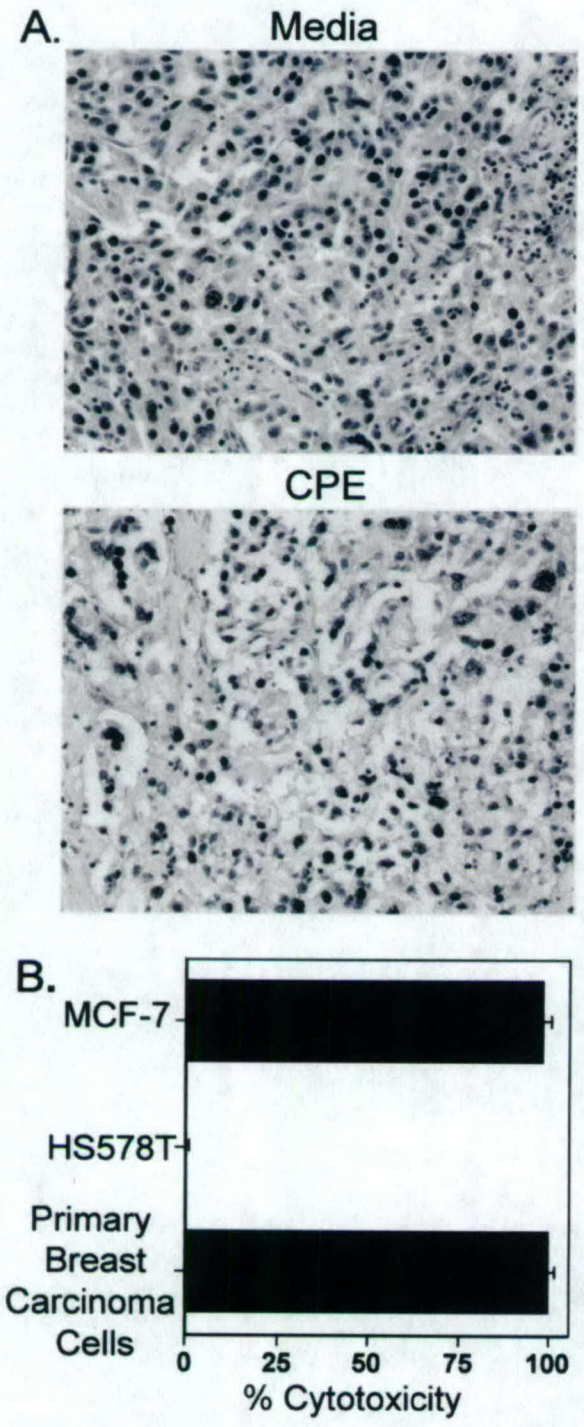


Figure 4. Sensitivity of primary breast tumor to CPE-mediated cytolysis *in vitro*. **A:** Freshly resected primary breast tumor tissue samples of approximately 5 to 10 mm^3 were divided into two equal pieces and incubated with or without 10 μg CPE in DMEM supplemented with 10% fetal bovine serum for a period of 12 hours. Tissues were subsequently fixed in 10% neutral buffered formalin, paraffin embedded, sectioned, and stained with H&E. Sections were visualized by light microscopy (×200). **B:** Primary breast carcinoma cells were isolated from freshly resected breast tumor using the epithelia-specific antibody BerEP4 conjugated to magnetic beads as described in Materials and Methods. Cells were aliquoted equally into the wells of a 6-well plate and treated with or without CPE at a final concentration of 2 μg/ml for 60 minutes. Percent cytotoxicity was determined by comparison of non-ballooned cell counts within a demarcated 1-cm^2 grid at 0 and 60 minutes following the addition of CPE. The breast cancer cell lines MCF-7 and HS578T were included as positive and negative controls, respectively.

mon problem facing most therapies is the development of a neutralizing antibody response preventing their repeated use. Although elevated titers of anti-enterotoxin antibodies developed following CPE ingestion have been found to provide no protection for human subjects against the effects of subsequent ingestion of CPE, careful studies need to be performed to accurately determine the presence, and nature of the immune response against CPE when administered by intratumoral, intraductal, and systemic routes.[17]

Another problem in the effective use of CPE as an anti-tumor agent is the inability to penetrate solid tumors. We found that intratumoral administration of CPE to the center of breast cancer xenografts resulted in significant necrosis, but left the boundaries of several tumors intact (data not shown). This suggests that CPE may have been largely restricted close to the point of injection and was unable to diffuse completely throughout the tumor. It is possible that alternative slow release formulations of CPE, such as liposomal preparations[18] or polymers carrying the drug,[19] would result in greater tumor penetration and improve therapeutic efficacy.

Systemic toxicity is an important concern for any new drug therapy. Although CPE-mediated cytolysis is specifically targeted against cells expressing CLDN 3 and 4, rendering many tissues immune to its effects, many other tissues such as prostate, lung, and the gastrointestinal tract express these proteins. Thus, systemic delivery of CPE would result in significant toxicity. This has been documented in mice where administration of doses as low as 0.1 mg/kg i.p. elicited symptoms associated with CPE-induced toxicosis such as immobility and loss of appetite.[20] In our studies, intratumoral administration of 0.5 mg/kg resulted in a significant reduction in tumor volume without any signs of CPE-induced toxicosis (Figure 3B). Under the same conditions, administration of the same dose i.p. was toxic and had no effect on tumor volume (data not shown). Thus, local delivery methods may be most appropriate for the use of native CPE.

To deliver CPE locally we are currently exploring the use of intraductal administration. Administering therapeutics through the ductal network allows more direct access to both primary breast tumors and pre-neoplasias while greatly reducing systemic exposure. Although local delivery of CPE into the breast duct may circumvent systemic toxicity, it could also cause death of normal ductal cells, since they express low but detectable levels of CLDN 3 and 4 (Figure 1A). Our preliminary studies addressing this issue show that normal rat breast epithelium appears to be relatively resistant to the effects of CPE. On gross examination of stained whole mounts of untreated rat mammary glands and those treated with CPE, no differences were observed. More detailed examination by microscopic analysis of H&E stained sections did show evidence of prior damage in CPE-treated glands in the form of gland atrophy and hemosiderin deposition; however, the ductal architecture of the treated glands remained intact. The apparent reduced sensitivity of normal breast epithelium to CPE-mediated cytolysis was not due to reduced susceptibility of rat cells to CPE, since treatment of two rat mammary cancer cell lines estab-

lished from *N*-nitroso,*N'*-methylurea-induced tumors expressing CLDN 3 and 4 resulted in rapid and virtually complete cytolysis in a dose-dependent fashion similar to that seen in human breast cancer cell lines (unpublished data). These preliminary studies suggest that normal mammary epithelial cells may be less susceptible to the effects of CPE when delivered by the intraductal route despite the expression of CLDN 3 and 4. The apparent reduced sensitivity of normal mammary epithelium to CPE could be due to the formation of more efficient tight junctions by normal mammary epithelium that limit CPE access to its receptors, and/or due to the lower expression of CLDN 3 and 4 in normal epithelium compared to tumor cells. This phenomenon has been previously documented in cell culture studies where CPE added to the apical domain of cells had a greatly reduced cytolytic effect than when added basolaterally.[3] As carcinoma cells invade the breast duct, both the apical and basolateral domains of the cell become exposed to intraductally delivered agents. Further, these cells lose cell polarity, which also may result in an altered distribution of the receptors along the cell surface. In addition, cancerous lesions are known to have reduced cell cohesion.[21,22] These factors would allow CPE to better contact its receptors and may explain the large differential in cytolytic effect of CPE on tumor cells compared to normal ductal epithelium, warranting detailed investigation into this delivery method.

Although the clinical application of CPE faces several challenges, it has several potential advantages as well. Currently, there are no known inhibitors of CPE. CPE-mediated cytolysis requires only the single step of CPE binding to its receptor. This is in contrast to other toxins and pro-drugs such as anthrax toxin and 5-fluorocytosine, which require additional enzymatic activation steps. Thus, the simplicity of CPE-mediated cytolysis may result in increased efficacy and reduced opportunity for the development of resistance. In addition, the documented ability of CPE to down-regulate the tight junction barrier through binding to CLDN 3 and 4[23] may enhance the anti-tumor effect of other treatment modalities. The local delivery of native CPE may be useful in the treatment of pre-neoplastic lesions such as ductal carcinoma *in situ* and in neo-adjuvant settings such as the locoregional control of locally advanced breast carcinoma, as well as in tumor down-staging to allow breast conservation therapy. Taken together, these data provide evidence to suggest that CPE may have potential in the treatment of breast cancer.

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